Portulaca oleracea extract increases lecithin:cholesterol acyltransferase and paraoxonase 1 activities and enhances reverse cholesterol transport in streptozotocin-induced diabetic rat

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ABSTRACT

Background: Plant extracts are generally assumed to be more acceptable and less hazardous than synthetic compounds and could be alternative antidiabetic treatments. *Portula caoleracea* has been used as one of the traditional edible and medicinal plant in Algeria to treat diabetes. The aim of the present study was to determine the effects of lyophilized aqueous extract of *Portulaca oleracea* on high-density lipoproteins composition, paraoxonase (PON1) and lecithin:cholesterol acyltransferase (LCAT) activities in streptozotocin-induced diabetic rat. **Methods:** Diabetes was induced intraperitonially by a single injection of streptozotocin (STZ) (60mg/kg bw). Twelve diabetic rats, weighing 263 ± 5g, were divided into two groups fed a casein diet supplemented or not with *Portulaca oleracea* extract (1g/kg bw), for 4 weeks. **Results:** At d28, in *Portulaca oleracea* treated *vs* untreated diabetic group, glycemia, serum total cholesterol (TC), triacylglycerols (TG) and phospholipids (PL) concentrations were decreased significantly ($p < 0.05$). The hypolipidemic effect induced by *Portulaca oleracea* extract was due to the reduction of total cholesterol (TC) in LDL-HDL_1 (-51%) and C-HDL_3 (-21%). *Portulaca oleracea* treatment improved PON1 and LCAT activities by 48%. HDL_3-UC (acyl group acceptor) and -PL (enzyme substrate) were diminished respectively by 47% and 82%, whereas HDL_2-CE concentrations (product of LCAT reaction) were increased by 44%. Moreover, HDL-C levels were found to be positively correlated with PON1 activity ($r = 0.96$, $p < 0.05$).Serum, LDL-HDL_1, HDL_2 and HDL_3 TBARS levels were respectively, 2.9-, 2.6-, 2.4- and 2.8-fold lower in *Portulaca oleracea* treated than untreated diabetic groups. *Conclusion:* These findings reflect the potential antihyperglycemic and hypolipidemic of *Portulaca oleracea* extract, in STZ-induced diabetic rat. Moreover, Portulaca oleracea extract restores PON1 and ameliorates the reverse cholesterol transport (RCT) by enhancing LCAT activity, therefore could prevent many diabetic complications by reducing dyslipidemia and oxidative damage.

Keywords: Rats, Streptozotocin, *Portulaca oleracea*, Cholesterol, PON1, LCAT, apo A-I, lipoprotein peroxidation

BACKGROUND

Diabetes mellitus (DM) is increasingly common metabolic disorder and one of the five leading causes of death in the world. Such disorders lead to various profound secondary complications like as, atherosclerosis, myocardial infarction, retinopathy and nephropathy.[1] DM is associated with an elevated level of oxidative stress, increased susceptibility to coronary heart disease, and reductions in Lecithin–cholesterol acyltransferase (LCAT)[2] and paraoxonase activities.[3] Lecithin–cholesterol acyltransferase (LCAT, EC 2.3.1.43) is one of the major modulators of plasma high-density lipoprotein cholesterol (HDL-C) and plays a central role in the reverse cholesterol transport (RCT) process.[4] LCAT was first described as a plasma enzyme responsible for the formation of cholesteryl esters in plasma. Human paraoxonase (EC.3.1.8.1, aryldialkylphosphatase) is a calcium-dependent esterase closely associated with high density lipoprotein (HDL)-containing apolipoprotein A-I (apo A-I),[5] which has been shown to confer antioxidant properties to HDL. Patients with DM, have characteristic abnormalities of plasma lipids and lipoprotein concentrations that almost certainly play a

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DOI: 10.5530/pj.2014.3.1

significant role in the increased risk for coronary heart disease.[6] This dyslipidemia is characterized by higher plasma triglyceride levels, both in the fasting and the postprandial state, reduced levels of high-density lipoprotein cholesterol (HDL-C), and abnormal low-density lipoprotein (LDL) particles.[7] Indeed, plasma lipoprotein metabolism is influenced by several factors, such as hyperglycemia, one of the main features of diabetes, results in non-enzymatic glycation of plasma proteins, including apo A-I, the most abundant apos in HDL. Glycation affects the structure of apoA-I and its ability to activate LCAT, the key enzyme in the reverse cholesterol transport.[8] Moreover, LCAT is glycated and oxidized as a result of chronic hyperglycemia, the mechanism of paraoxonase reduction in oxidative stress status is not clearly known. However, it is suspected that ROS overproduction leads to increased deactivation of paraoxonase.[9] Several studies also reported that LCAT activity was lower in both type 1 and type 2 diabetic patients than in control subjects.[10] Another study reported significantly higher malondialdehyde concentration and lower paraoxonase activity in patients with type 2 diabetes, illustrating a negative correlation between paraoxonase activity and lipid peroxidation.[11] Traditional medicines derived mainly from plants have played and still plays major role in the management of DM.[12] *Portulaca oleracea* L (from *Portulacaceae* family) locally named "Redjila" is widely used in Algerian popular medicine, shows some benefits in the treatment of diabetes, cardiovascular diseases and enhancing immunity.[13] Tender stems and leaves are usually eaten raw, alone or with other greens. They are also cooked or pickled for consumption.The traditional use of *Portulaca oleracea* in the treatment of diabetes have not yet been studied in great detail. Decoction and infusion are the methods mostly used for preparation of the traditional medicine. Recent research demonstrated that this plant is a good source of compounds with a positive impact in human health. Those compounds include omega-3 fatty acids, phenolics, coumarins and alkaloids. The aqueous extracts of *Portulaca oleracea* show no cytotoxicity or genotoxicity, and have been certified safe for daily consumption as a vegetable.[14] Thus, the present study was designed to assess the effects of the lyophilized aqueous extract of *Portulaca oleracea* on lipid profile, lipoprotein peroxidation, LCAT and paraoxonase activities, in the streptozotocin-induced diabetic rat.

MATERIAL AND METHODS

Plant material

Portulaca oleracea was collected in Southern of Algeria (Touggourt), between March and April 2012, identified taxonomically and authenticated by the Botanical Research Institute of Oran University (voucher specimen number *Po1965*). The plant material was stored at room temperature in a dry place before use. Fresh aerial parts (leaves) of the plant were dried at ambient temperature (24˚C) for 7 days and ground to a powder. The *Portulaca oleracea* extract was prepared as follows: 50 g of the powdered aerial parts was refluxed at 60-70˚C in 500 ml distillated water for 30 minutes and the decoction was filtered with cotton wool. The filtrate was concentrated at 65˚C by a rotavapor (BuchiLabortechnink AG, Postfach, Switzerland) under a reduced pressure and frozen at -70˚C before lyophilization (Christ, alpha 1-2 LD). The crude yield of the lyophilized extract was approximately 25% (wt/wt). It was stored at ambient temperature until further use.

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out by using standard procedure described by Harborne.[15]

Animals and experimental design

Male Wistar rats (Iffa Credo, l'Arbresle, Lyon, France), weighing 263±5g were housed under standard environmental conditions (23±1˚C, 55±5% humidity and a 12 h light/dark cycle) and maintained with free access to water and a standard diet *ad libitum*. The General Guidelines on the Use of Living Animals in Scientific Investigations[16] were followed, and Institutional Animal Ethical Committee (CPCSEA Reg. No. 12/314/2012) approval for experimental protocol was obtained.

Induction of diabetes in rats

Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (Sigma, St Louis, Mo, USA) at a dose of 60 mg/kg bw STZ was dissolved in 0.05 mol/L cold sodium citrate buffer, pH 4.5 immediately before use. After 48 h, hyperglycemia was confirmed using a Glucometer (Accu-Chek® active, Germany). Only animals with fasting blood glucose levels greater than 16 mmol/Lwere considered diabetic and then included in this study. Diabetic rats (n=12) were randomly divided into two groups. The untreated group received a casein diet and the treated group received the same diet supplemented and mixed with the *Portulaca oleracea* extract (0.1%), for 4 weeks. The ingredient composition of the diets is shown in Table 1.

Blood samples

After the 4 weeks of the experiment, the rats were fasted overnight and anesthetized with chloral hydrate 10% (3 ml/kg bw) and euthanized with an overdose. Blood was

Table 1. Ingredient composition of the diets fed to rats (g/kg diet)[1]

Ingredients	D	DPo
Casein[2]	200	200
Corn starch[3]	590	573.4
Sunflower oil[4]	50	50
Sucrose[5]	50	50
Cellulose[2]	50	50
Vitamin mix[6]	20	20
Mineral mix[7]	40	40
Portulaca oleracea extract[8]	0	1

[1]Diets were isoenergetic (16.28 MJ/kg) and given in powdered form.
[2]PROLABO (Paris, France).
[3]ONAB (Sidi Bel Abbès, Algeria).
[4]CEVITAL (Béjaïa, Algeria).
[5]ENASUCRE (Sfisef, Algeria).
[6]UAR 200 (Villemoisson, 91360). Vitamin mixture provided the following amounts (mg/kg diet): vitamin A, 39 600 UI; vitamin D_3, 5000 UI; vitamin B_1, 40; vitamin B_2, 30; vitamin B_3, 140; vitamin B_6, 20; vitamin B_7, 300; vitamin B_{12}, 0.1; vitamin C, 1600; vitamin E, 340; vitamin K, 3.80; vitamin PP, 200; choline, 2720; folic acid, 10; paraaminobenzoic acid, 180; biotin, 0.6; and cellulose, qsp, 20 g.
[7]UAR 205B (Villemoisson, 1360, Epinay/S/Orge, France). Mineral mixture provided the following amounts (mg/kg diet): $CaHPO_4$, 17 200; KCl, 4000; NaCl, 4000; MgO_2, 420; $MgSO_4$, 2000; Fe_2O_3, 120; $FeSO_4$, $7H_2O$, 200; $MnSO_4$, $H2SO_4$, H_2O, 98; $CuSO_4$, $5H_2O$, 20; $ZnSO_4$, 80; $CuSO_4$, 80; CuSO4, $7H_2O$; and KI, 0.32.
[8]Prepared in our laboratory as previously described.

obtained from the abdominal aorta of rats and collected into dried tubes and centrifuged at 1000g for 20 minutes at 4˚C. Serum samples were stored at -70˚C until use.

Lipoprotein separation

Serum LDL-HDL_1were isolated by precipitation using $MgCl_2$ and phosphotungstate (Sigma Chemical Company, France) by the method of Burstein *et al.*[17] HDL_2 and HDL_3 were separated by precipitation according to the method of Burstein *et al.*[18] using $MgCl_2$ and dextran sulfate (Sigma Chemical Company, France).

Biochemical analysis

Blood glucose levels were determined as described above. Glycosylated haemoglobin (HbA_1C) was estimated by ion exchange chromatography method (KitBiocon, Germany). Serum urea and creatinine were analyzed by enzymatic methods (KitsBiocon, Germany) and apo A-I concentrations were determined by immuno-turbidimetric method (kit Orion Diagnostica, Spain). Total cholesterol (TC) Triacylglycerol (TG) and phospholipids (PL) of serum and each fraction were determined with enzymatic method kit (Kits Spinreact, Girona Spain). In HDL_2 and HDL_3 fractions, unesterified cholesterol (UC) contents were determined using enzymatic method (Kit Wako, Germany). Esterified cholesterol (EC) concentrations were obtained by calculating the difference between TC and UC values. Cholesteryl esters (CE) levels were estimated as 1.67 times the esterified cholesterol content. Protein concentrations were measured according to the method of Lowry *et al.*,[19] using bovine serum albumin (Sigma Chemical Company, St Louis, MO, USA) as a standard.

Lipid peroxidation assay

Thiobarbituric acid-reactive substances (TBARS) were determined in serum and lipoproteins fraction according to the method of Quantanilha *et al.*[20] One milliliter of diluted serum or each fraction was added to 2 ml of thiobarbituric acid (TBA) (final concentration, 0.017 mmol/l), plus butylated hydroxytoluene (concentration, 3.36 µmol/l) and incubated for 15 min at 100˚C. After cooling and centrifugation, the absorbance of supernatant was measured at 535 nm.

Lecithin:cholesterol acyltransferase (LCAT) activity assay

Serum LCAT activity was assayed by the conversion of unesterified [^{3}H] cholesterol to esterified [^{3}H] cholesterol, according to the method of Glomset and Wright,[21] modified by Knipping.[22] Cholesterol and egg phosphatidylcholines (PC) were used for the preparation of liposomes. Specifically, 2 mg cholesterol and 16 mg egg PC in chloroform-methanol (2:1, vol/vol) were evaporated to dryness under nitrogen stream. After adding 1.0 ml of (10 mmol/l Tris-HCl, 150 mmol/l NaCl and 1.0 mmol/l EDTA, pH 7.4), the solution was sonicated for 30 minutes at 100 W and 25˚C. LCAT activity was determined using liposomes as substrate. The cholesterol esterifying activity was expressed as nanomoles of esterified cholesterol/h/ml of Serum.

Paraoxonase (PON1) activity assay

Serum PON1 activity was estimated spectrophotometrically by the method of Schiavon*et al.*[23] Briefly, the assay mixture consisted of 500 µl of 2.2 mmol/l paraoxone substrate in 0.1 mol/l tris-HCl buffer, pH 8, containing 2 mmol/L $CaCl_2$ and 50 µl of fresh serum. The absorbance was monitored at 405 nm, at 25˚C. The PON1 activity was expressed in international units (IU). One IU was defined as 1 µmol of p-nitrophenol which was formed/min/L.

Statistical analysis

All data are presented as means ± SEM of 6 rats per group. Statistical analysis was carried out by the Student "t" test. The calculations were performed using STATISTICA (Version 10, StatSoft, Tulsa, Okla). Value of $P<0.05$ was

considered to be statistically significant. Linear regression analysis was used to determine correlation coefficient between PON1 activity and HDL-C levels.

RESULTS

Phytochemical screening

Preliminary phytochemical screening of *Portulaca oleracea* aqueous extract revealed the presence of tannins, flavonoids, phenolics, carbohydrates, steroids, saponins, aminoacids and proteins (Table 2).

Glycemia, glycosylated hemoglobin, serum urea, and creatinine concentrations

In the diabetic rats, daily administration of *Portulaca oleracea* extract induced a significant decrease of glycemia and glycosylated haemoglobin levels (Table 3). However, serum urea and creatinine concentrations were reduced by 27% and 31%, respectively.

Serum lipids concentrations and atherogenicity ratios

Portulaca oleracea treatment lowered significantly serum TC (-38%) and LDL-HDL_1-C (-51%), whereas HDL-C concentrations were increased by 26% (Table 4). Serum TG and PL levels were enhanced respectively by 53% and 62% in *Portulaca oleracea*-treated compared to untreated diabetic group. TC/HDL-C and LDL-HDL_1-C/HDL-C ratios were respectively, 2.2- and 2.7-fold lower in Portulaca oleracea treated than untreated diabetic group.

Serum HDL_2 and HDL_3 amounts and compositions

The amounts of lipoproteins which represents the sum of apolipoproteins (apos), UC, CE, TG and PL contents reduced by -29% in HDL_2, in *Portulaca olreracea* treated *vs* untreated diabetic group (Figure 1). The contents of apos and UC were similar in the both groups, whereas CE concentrations were 1.5-fold higher. Inversely, TG and PL levels were 1.4-, 1.3-fold lower, in *Portulaca oleracea*-treated *vs* untreated diabetic group.

Portulaca oleracea treatment reduced significantly HDL_3amount (-61%) (Figure 2). However, UC and PL concentrations were respectively decreased by 48% and 83% in *Portulaca oleracea* treated compared with untreated diabetic rats. In addition, apos, CE and TG contents were similar in the both groups.

Serum and lipoproteins lipid peroxidation

Lipid peroxidation was significantly decreased in *Portulaca oleracea* group. Serum, LDL-HDL_1, HDL_2 and HDL_3 TBARS contents were respectively, 2.9-, 2.6-, 2.4- and 2.8-fold lower in *Po* treated than untreated diabetic groups (Table 5).

Serum apo A-I concentrations, LCAT and PON1 activities

Serum apo A-I concentrations tended to be higher but not significantly **(**Table 6**)**. However, in *Portulaca oleracea*-treated *vs* untreated diabetic group, a higher LCAT and PON1 activities were noted (+48%).

DISCUSSION

Evaluation of plant products to treat *diabetes mellitus* is of growing interest as they contain many bioactive substances with therapeutic potential. As mentioned above, various phytotherapeutic products (phenolics, coumarins and alkaloids) are already used; and convey satisfactory results. In essence, *Portulaca oleracea* was widely used in traditional medicine for their various pharmacological

Table 2. Preliminary phytochemical components screening of *Portulaca oleracea* extract

Phytochemical Compounds	Presence
Tannins	+++
Flavonoids	++
Phenolics	++
Carbohydrates	+
Steroids	+
Saponins	+
Proteins and amino acids	+

All tests were performed six times. +++ = appreciable amount (positive within 5 mins.); ++ = moderate amount (positive after 5 mins); + = trace amount (positive after 10 mins).

Table 3. Glycemia, glycosylated hemoglobin, urea, and creatinine concentrations

	D	D*Po*
Glycemia (mmol/L)	27.09 ± 2.29	9.54 ± 2.18***
HbA1c (%)	10.11 ± 0.38	5.94 ± 0.58**
Urea (mmol/L)	8.16 ± 1.10	5.96 ± 0.45*
Creatinine (µmol/L)	61.03 ± 8.06	42.09 ± 8.41*

Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test.
**$p<0.01$, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)
***$p<0.001$, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)

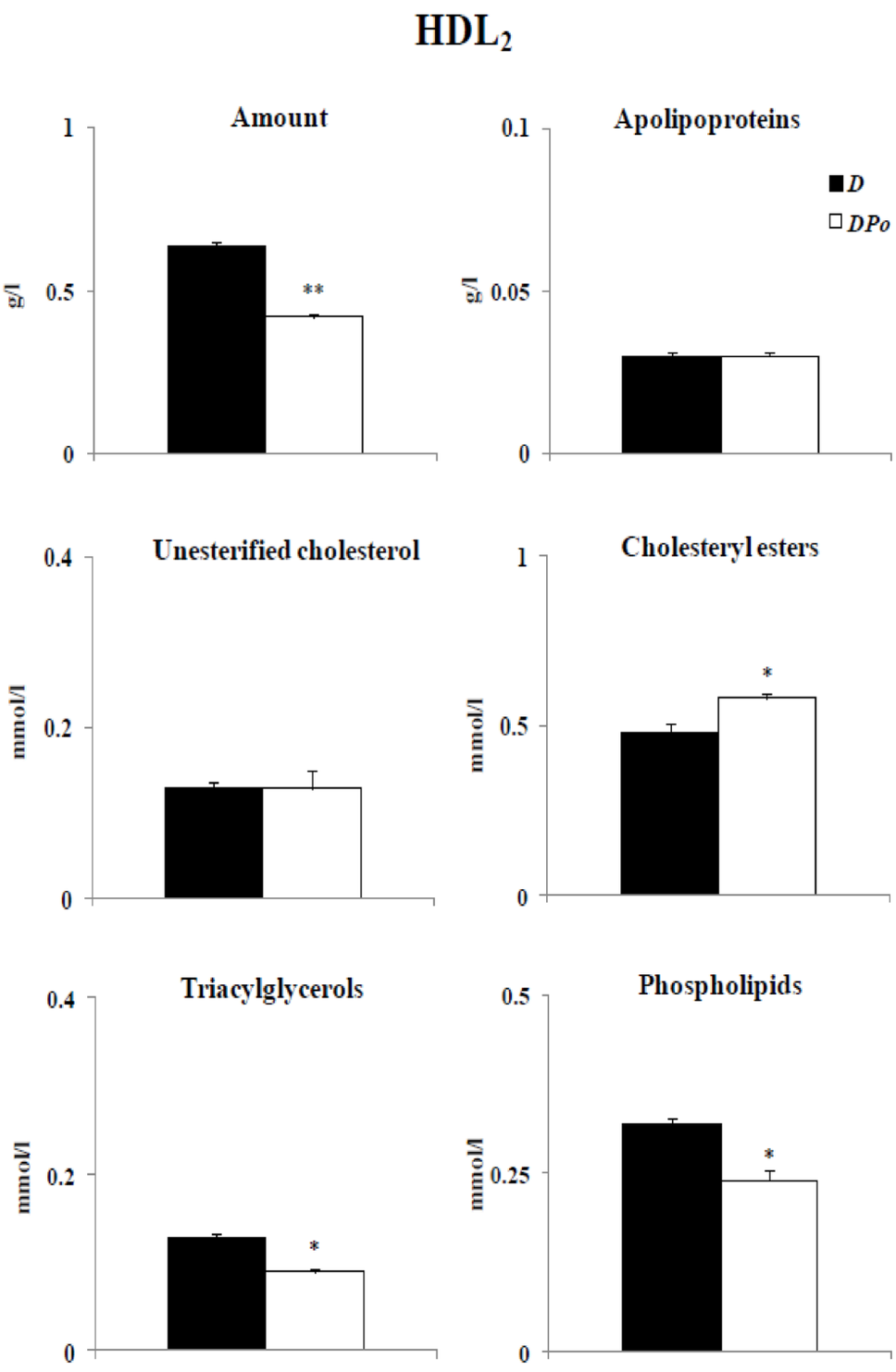

Figure 1. Serum HDL2 amounts and compositions.
Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test.
*p<0.05, diabetic rats treated with the *Portulaca oleracea* extract (DPo) vs untreated diabetic rats (D).
**p<0.01, diabetic rats treated with the *Portulaca oleracea* extract (DPo) vs untreated diabetic rats (D).

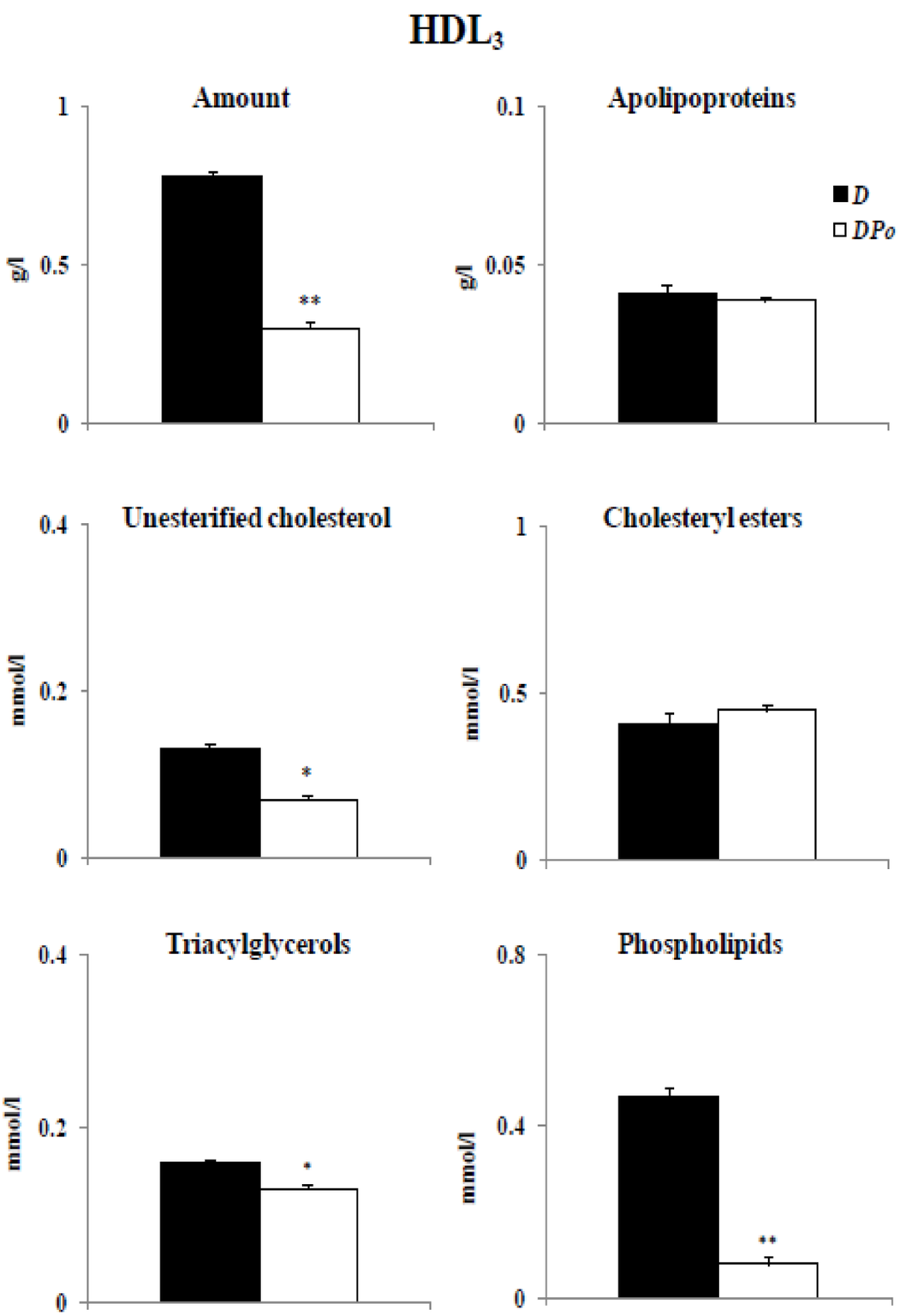

Figure 2. Serum HDL3 amounts and compositions.
Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test.
*p<0.05, diabetic rats treated with the *Portulaca oleracea* extract (DPo) vs untreated diabetic rats (D).
**p<0.01, diabetic rats treated with the *Portulaca oleracea* extract (DPo) vs untreated diabetic rats (D).

Table 4. Serum lipids concentrations (mmol/L) and atherogenicity ratios

	D	D*Po*
Serum-TC	2.00 ± 0.21	1.24 ± 0.19*
LDL-HDL_1-C	0.73 ± 0.04	0.35 ± 0.09*
HDL-C	0.43 ± 0.01	0.58 ± 0.06*
TC/HDL-C	4.74 ± 0.09	2.19 ± 0.56*
LDL-HDL_1-C/HDL-C	1.71 ± 0.14	0.62 ± 0.22*
Serum-TG	0.96 ± 0.02	0.45 ± 0.06***
Serum-PL	1.31 ± 0.07	0.49 ± 0.09***

Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test.
*p<0.05, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)
***p<0.001, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)

Table 6. Apolipoprotein (apo A-I), lecithin:cholesterol acyltransferase (LCAT) and paraoxonase (PON1) activities

	D	D*Po*
apo A (g/L)	1.19 ± 0.80	2.14 ± 0.32
LCAT(nmol/mL/h)	11.60 ± 3.17	21.90 ± 03.16***
PON1 (IU)	4.30 ± 0.62	8.44 ± 0.97**

Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test. Values were considered to be different at p< 0.05 and p< 0.01.
**p<0.01, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)
***p<0.001, diabetic rats treated with *Portulaca oleracea* extract (DPo) *vs* untreated diabetic rats (D)

effects. In this paper, we had experimentally investigated the anti-diabetic effect of *Portulaca oleracea* lyophilised aqueous extracts, with special focus on their impact on High-density lipoprotein composition and paraoxonase, LCAT activities.

Previous studies have shown that polysaccharides, flavonoids, steroids and alkaloids present in the medicinal plants can justify the properties of some herbs in decreasing the blood sugar.[24] At day 28, our results showed that the treatment with *Portulaca oleracea* extract decreased significantly glycemia value. This hypoglycemic effect could be directly related to the polyphenolic compounds of the extract [Table 2]. Glycosylated haemoglobin is one of those glycosides compounds in particular that reflects average blood glucose in patients 2 or 3 months before blood collection.[25] The results obtained from the measurement of HbA_1C show a clear deference between untreated diabetic (10%) and *Portulacaoleracea*-diabetic groups (6%). This result was concomitant with the hypoglycemic effect induced by *Portulaca oleracea* extract. It is well known that diabetes leads to alterations of renal function essentially due to an accumulation of advanced glycated end products (AGE).[26] Diabetic nephropathy in uncontrolled

Table 5. TBARS levels in serum and lipoproteins fraction (nmol/mL)

	D	D*Po*
Serum	12.91 ± 01.43	4.51 ± 1.59**
LDL-HDL_1	3.25 ± 00.11	1.26 ± 0.46**
HDL_2	1.31 ± 00.39	0.54 ± 0.10*
HDL_3	1.74 ± 00.23	0.63 ± 0.11**

Values are mean ± SEM of 6 rats per group. Statistical analysis was performed using the student't' test.
*p<0.05, diabetic rats treated with *Portulaca oleracea* extract (**D*Po***) *vs* untreated diabetic rats (**D**)
p<0.01, diabetic rats treated with *Portulaca oleracea* extract (D*Po***) *vs* untreated diabetic rats (**D**)

diabetes is a serious micro-vascular complication which results in increased blood urea and creatinineconcentrations.[27] Interestingly, the higher levels of urea and creatinine in diabetic rats are diminished significantly after the administration of *Portulaca oleracea*, which could be a result of improved renal function due to the reduced plasma glucose concentration and subsequent glycosylation of renal basement membranes.

Diabetes mellitus is related to a hyperlipidemia and leads to serious anomalies in lipids composition and concentration.[28] These anomalies can induce cardiovascular diseases.[29] The high level of total cholesterol in the blood could be seen as a major risk factor generating coronary heart disease.[30] It is known that the increase in serum lipids in STZ-induced diabetic rats played an important role in such pathology.[31] In our study, *Portulaca oleracea* aqueous extract supplementation prevented hyperlipidemia by reducing the levels of cholesterol in LDL-HDL_1 and increasing serum HDL_2-C. It could be suggested that these effects were explained by presence of flavonoids in *Portulaca oleracea* extract. Since these compounds have effectiveness in reducing blood lipid, as an anti-oxidative, in assimilating cholesterol, inhibiting thrombosis and dilating the coronary artery[32]. In some other studies, supplementation with omega-3 fatty acids resulted in significant increase in the HDL cholesterol.[33] *Portulaca oleracea* is a rich source of Omega 3 fatty acids and it has been reported that Omega 3 fatty acids reduce LDL-C.[34] Moreover, TC/HDL-C ratio or LDL-C/HDL-C which are a marker of dyslipidemia was reduced in *Portulaca oleracea*-diabetic group than untreated diabetic group. These decreased ratios are predictors of coronary risk.[35] High density lipoprotein (HDL) is one of the most important independent protective factors for the arteriosclerosis which underlies coronary heart disease. Flavonoids may also decrease cholesterol absorption by increasing the excretion of bile acids.[36] Increased HDL-C level can also facilitate the transport of cholesterol from tissues to liver.[37] The possible mechanism of this activity may result from

the enhancement of lecithin:cholesteryl acyltransferase (LCAT).[38] In *Portulaca oleracea* treated compared with untreated diabetic group, LCAT activity was increased and the concentrations of plasma apo A-I tended to be higher but not significantly. Recent research findings from animal and human studies have revealed a potential beneficial role of LCAT in reducing atherosclerosis.[39] In the present study, *Portulaca oleracea* treatment decreased significantly HDL_3-PL (the enzyme substrate) and HDL_3-UC (acyl group acceptor) concentrations and increased HDL_2-CE levels (product of enzymatic reaction). These findings showed that *Portulaca oleracea* extract enhanced LCAT activity ensuring HDL_3-HDL_2 conversion and the subsequent hepatic uptake.

The previous report of Sinha *et al*.[40] suggests that, the hypolipidemic activity may be attributed to inhibition of oxidative stress. In addition, our result showed that TBARS levels in serum, LDL-HDL_1, HDL_2 and HDL_3 were decreased significantly in *Portulaca oleracea*-treated diabetic rats compared to untreated diabetic rats. These results indicated that *Portulaca oleracea* extract might protect the plasma the cytotoxic action and oxidative stress of streptozotocin. In fact, we know that a low HDL concentration is usually accompanied by a reduction in PON1 activity or concentration in diabetic patients.[41] In this data, higher paraoxonase activity and lower TBARS concentration in *Portulaca oleracea*-treated diabetic rats, illustrating a positive correlation between paraoxonase activity and serum HDL-C levels ($r = + 0.96$, $p<0.01$). Several studies have indicated that PON1 can prevent lipid peroxide accumulation on LDL.[42] Recently studies demonstrated that PON1 is an important antioxidant enzyme and is responsible for antioxidant effects of HDL-C[43] and its activity can be modified by factors such as diet and lifestyle.[44] Therefore, the increased PON1 activity is shown in our study and could be due to polyphenols contained in *Portulaca oleracea*. Polyphenols have antioxidant activity and also, modulates gene expression of PON1 leading to increased PON1 activity.[45]

CONCLUSIONS

These findings reflect clearly the potential antihyperglycemic and hypolipidemic of *Portulaca oleracea* aqueous lyophilized extract in streptozotocin-induced diabetic rat. Moreover, *Portulaca oleracea* extract prevents lipoprotein oxidationby enhancing PON1 activity, and ameliorates the reverse cholesterol transport by enhancing LCAT activity, thereby providing protection against diabetic dyslipidemia.

ABBREVIATIONS

Apo A-I: Apolipoprotein A-I; CE: cholesteryl esters; DM: *Diabetes mellitus*; HbA_1C: Glycosylated haemoglobin; HDL-C: High density lipoprotein-cholesterol; LCAT: Lecithin:cholesterol acyltransferase; LDL-C: Low density lipoprotein-cholesterol; PON1: Paraoxonase 1; PL: Phospholipids; *Po: Portulaca oleracea*; STZ: Streptozotocin; TBARS: Thiobarbituric acid-reactive substances; TC: Total cholesterol; TG: Triglycerides; UC: Unesterified cholesterol.

ACKNOWLEDGEMENTS

This research was supported by the Algerian Ministry of Higher Education and Scientific Research.

COMPETING INTERESTS

All authors of this research have no conflict of interest related with employment, consultancies, stock ownership, grants or other funding.

REFERENCES

1. Shen X: Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase.*Can Jof PhysiolPharm* 2010, 88(3):241–248.
2. Mackness B, Durrington PN, Boulton AJ, Hine D, Mackness MI:Serum paraoxonase activity in patients with type 1 diabetes compared to healthy controls.*Eur J Clin Invest* 2002, 32:259–64.
3. Guan JZ, Tamasawa N, Murakami H: HMG CoA reductase inhibitor, simvastatin improves reverse cholesterol transport in type 2 diabetic patients with hyperlipidemia.*J AtherThromb*2008, 15:20–25.
4. Jonas A:Lecithin cholesterol acyltransferase.*BiochimBiophysActa* 2000, 1529:245–246.
5. Vekic J, Kotur-Stevuljevic J, Jelic-Ivanovic Z, Spasic S, Spasojevic-Kalimanovska V, Topic A:Association of oxidative stress and PON1 with LDL and HDL particle size in middle-aged subjects.*Eur J Clin Invest* 2007,37:715–723.
6. Chen J, Li W L, Wu J L, Ren R B, Zhang H Q:Hypoglycemic effects of a sesquiterpene glycoside isolated from leaves of loquat (*Eriobotrya japonica*).*Phytomedicine* 2008, 15(1–2):98–102.
7. Vergès B: Lipid modification in type 2 diabetes: the role of LDL and HDL.*FundamClinpharmacol* 2009, 23:681–685.
8. Nobecourt E, Davies MJ, Brown BE:The impact of glycation on apolipoprotein A-I structure and its ability to activate lecithin:chol esterolacyltransferase.*Diabetologia* 2007, 50:643–653.
9. Isik A, Koca SS, Ustundag B, Celik H and Yildirim A:Paraoxonase and arylesterase levels in rheumatoid arthritis.*ClinRheumatol* 2007, 26(3): 342–348.
10. Dirican M, Serdar Z, Sarandöl E, Sürmen-Gür E:Lecithin: cholesterol acyltransferase activity and cholesteryl ester transfer rate in patients with *diabetes mellitus.Turk J Med Sci*2003, 33:95–101.
11. Rasic-Milutinovic Z, Popovic T, Perunicic-Pekovic G, Arsic A, BorozanS and Glibetic M:Lower serum paraoxonase-1 activity is

related to linoleic and docosahexanoic fatty acids in patients with type 2 diabetes.*Arch Med Res* 2012, 43(1):75–82.
12. Guo H, Ling W, Wang Q, Liu C, Hu Y, Xia M:Cyanidin 3-glucoside protects 3T3-L1 adipocytes against H_2O_2- or TNF-α-induced insulin resistance by inhibiting c-Jun NH_2-terminal kinase activation.*Biochempharmacol* 2008, 75:1393–1401.
13. An Sook L, Yun Jung L, So Min L, Jung Joo Y, Jin Sook K, Dae Gill K, Ho Su: *Portulaca oleracea* Ameliorates Diabetic Vascular Inflammation and Endothelial Dysfunction in db/db Mice.*Evid Based Complement Alternat Medicine* 2012, 10–1155.
14. Yen GC, Chen HY, Peng HH:Evaluation of the cytotoxicity, mutagenicity and anti-mutagenicity of emerging edible plants. *Food ChemToxicol* 2001, 39:1045–1053.
15. Harborne JB:Phytochemical Methods: A guide to modern techniques of plant analysis. *Chapman and Hall* 1998, 3:202–209.
16. Council of European Communities:Council instructions about the protection of living animals used in scientific investigations 1987. *Official J*. L358 of 18-12-1986,*Corrigendum Official J*. L117 of 05-05-1987.
17. Burstein M, Scholnick H R, Morfin R: Rapid method for isolation of lipoproteins from human serum by precipitation with polyanions. *Journal Lipid Res* 1970, 11:583–595.
18. Burstein M, Fine A, Atger V, Wirbel E, Girard-Globa A:Rapid method for the isolation of two purified subfractions of high density lipoproteins by differential dextran sulphate-magnesium chloride precipitation. *Biochem*1989, 71:741–746.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ:Protein measurement with the folin phenol reagent. *J BiolChem*1951, 193:265–75.
20. Quantanilha AT, Packer L, Szyszlo DJA, Racnelly TL, Davies KJA: Membrane effects of vitamin E deficiency bioenergetic and surface charge density of skeletal muscle and liver mitochondria. *Ann NY AcadSci* 1982, 393:32–47.
21. Glomset JA, Wright JL:Some properties of cholesterol esterifying enzyme in human plasma.*BiochemBiophysActa*1964, 89:266–271.
22. Knipping G:Isolation and properties of porcine lecithin: cholesterol acyltransferase. *Eur J Biochem*1986, 154:289–294.
23. Schiavon R, De Fanti E, Giavarina D, Biasioli S, Cavalcanti G, Guidi G:Serum paraoxonase activity is decreased in uremic patients.*ClinChimActa* 1996, 247:71–80.
24. Zarei A, Ashtiyani SC, Rasekh F: The effects of *Physalisalkekengi* extract on lipids concentrations in rats.*Persian. J Arak Med Sci* 2011, 14(55):36–42.
25. Alvarez-Garcia E:HbA1c, standardization and expression of results.*Endocrinologia y Nutricion* 2010, 57(5):177–181.
26. Yue KKM, Chung WS, Leung AWN, Cheng CHK: Redox changes precede the occurrence of oxidative stress in eyes and aorta, but not in kidneys of diabetic rats.*Life Sci* 2003, 73:2557–2570.
27. Bijlani RL, Vempati RP, Yadav RK:A brief but comprehensive lifestyle education program based on yoga reduces risk factors for cardiovascular disease and *diabetes mellitus*.*J AlternComplem Med* 2005, 11:267–274.
28. Cooperstin SJ, Watkin D:Action of Toxic Drugs on Islet Cells in the Islets of Langerhans. *Academic Press, New York* 1981, 387–425.
29. Al-Shamaony L, Al Khazraji MS, Twaij HA:Hypoglycemic effects of *Artemisia herba-alba*. Effect of a valuable extract on some blood parameters in diabetic animals. *J Ethnopharmacol* 1994, 43(3):167–171.
30. Brown GB, Xue-Qiao Z, Soccoand DE, Albert JJ: Lipid lowering and plaque regression. New insights into prevention of plaque disruption and clinical vents in coronary disease.*Circulation* 1993, 8:1781–1791.
31. Sharma S B, Balomajumder C, Roy P:Hypoglycemic and hypolipidemic effects of flavonoid rich extract from *Eugenia jambolana* seeds on streptozotocin induced diabetic rats.*Food ChemToxicol* 2008, 46(7):2376–2383.
32. Shao B, Guo H Z, Cui Y J: Simultaneous determination of six major stilbenes and flavonoids in Smilax china by high performance liquid chromatography.Journal Pharmaceut Biomed 2007, 44:737–742.
33. Mahmoodi MR, Kimiagar M, Mehrabi Y:The effects of omega-3 plus vitamin E and vitamin C plus zinc supplementations on plasma lipids and lipoprotein profile in postmenopausal women with type 2 diabetes.*Persian. NutrSci Food Technol* 2009, 4(3):1–14.
34. Chang CL, Seo T, Matsuzaki M, Worgall TS, Deckelbaum RJ:n-3 fatty acids reduce arterial LDL-cholesterol delivery and arterial lipoprotein lipase levels and lipase distribution. *ArteriosclerThrombVascBiol* 2009, 29:255–561.
35. Rajkumar M, Uttam KD, Debidas G:Attenuation of hyperglycemia and hyperlipidemia in streptozotocin-induced diabetic rats by aqueous extract of seed of *Tamarindusindica*.*Biol Pharm Bull 2005*, 28:1172–1176.
36. Devaraj S, Autret BC, Jialal I:Reduced-calorie orange juice beverage with plant sterols lowers C-reactive protein concentrations and improves the lipid profile in human volunteers. *Am J ClinNutr* 2006, 84(4):756–761.
37. Cesar TB, Aptekmann NP, Araujo MP, Vinagre CC, Maranhão RC: Orange juice decreases low-density lipoprotein cholesterol in hypercholesterolemic subjects and improves lipid transfer to high-density lipoprotein in normal and hypercholesterolemic subjects. *Nutr Res* 2010,30(10): 689–694.
38. Anila L, Vijayalakshmi NR: Flavonoids from *Emblicaofficinalis* and *Mangiferaindica*effectiveness for dyslipidemia. *J Ethnopharmacol* 2002, 79(1):81–7.
39. Rousset X, VaismanB, Amar M, Sethi AA, Remaley AT:Lecithin: cholesterol acyltransferase from biochemistry to role in cardiovas-cular disease. *CurrOpinEndocrinolDiabetesObes* 2009, 16:163–171.
40. Sinha M, Manna P, Sil PC:*Terminaliaarjuna* protects mouse hearts against sodium ßuoride-induced oxidative stress. *J Med Food* 2008, 11:733–740.
41. Ayub A, Mackness MI, Arrol S, Mackness B, Patel J, Durrington PN:Serum paraoxonase after myocardial infarction. *ArteriosclerThrombVascBiol* 1999, 19:330–5.
42. Abbott CA, Mackness MI, Kumar S, Boulton AJ, Durrington PN: Serum paraoxonase activity, concentration, and phenotype distribution in *diabetes mellitus*and its relationship to serum lipids and lipoproteins.*ArteriosclerThrombVascBiol* 1995, 15: 1812–1818.
43. Mahrooz A, Rashidi MR, Nouri M: Naringenin is an inhibitor of human serum paraoxonase (PON1): an in vitro study.*J Clin Lab Anal* 2011, 25(6):395–401.
44. Mackness B, Durrington P, McElduff P, Yarnell J, Azam N: Watt M. Low paraoxonase activity predicts coronary events in the Caerphilly Prospective Study. *Circulation* 2003, 107: 2775–2779.
45. Suehiro T, Nakamura T, Inoue M, Shiinoki T, Ikeda Y, Kumon Y: A polymorphism upstream from the human paraoxonase (PON1) gene and its association with PON1 expression.*Atherosclerosis* 2000, 150:295–298.

The Effects of Topical Carvacrol Application on Wound Healing Process in Male Rats

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ABSTRACT

Carvacrol containing products such as Origanum onites have been used as phytotherapeutic agents in the treatment of serious skin injury. It has been suggested that carvacrol is the active component of these herbs because of its anti-microbial property. With the anti-microbial activity and as an ingredient of these herbs, carvacrol is a promising molecule for the treatment of skin injury. In the present study, we have evaluated the efficacy of carvacrol on healing progress after excisional skin injury. Here, Wistar-Albino rats were divided into two groups and treated with carvacrol and vehicle. Carvacrol was administrated topically at a concentration of %12.5 for the 5 consecutive days after excisional skin injury. Tissue samples were harvested on days 3rd, 8th and 12th after injury. Significant beneficial effect of carvacrol was observed at the end of the experiment. In the acute phase of the injury, carvacrol treatment increased tissue granulation and decreased wound depth moderately. These effect of carvacrol was associated with increased TNF-α. However, at the second half of the experiment the elevated level of TGF-B_1 was observed as compared with control animals. The level of IL-1_β was increased in carvacrol treated animals only on day 8. Here, we provide evidence that carvacrol improves wound healing by regulating pro- inflammatory molecules TNF-α, IL-1_β and TGF-β_1.

Keywords: carvacrol; wound healing; TNF-α; IL-1_β; TGF-β_1; aromatic herbs.

INTRODUCTION

Carvacrol is an essential oil component of aromatic herbs such as Origanum onites.[1–4] In-vitro and in-vivo studies performed with carvacrol showed that it has diverse effects, including antimicrobial,[5,6] antiviral,[7] antifungal,[8–10] antioxidant,[11,12] and antispasmodic.[13–15] In this context, carvacrol is a promising molecule for the treatment of local skin injury.

Wound healing is defined as the completion of closure at the clinically wounded skin area. Wound healing processes develops from some pathophysiological events including haemostasis, inflammation, cellular proliferation, matrix synthesis and remodeling. It has been also shown that IL-1, TNF-α and TGF-β stimulate neutrophile migration, fibroblast activation and reepithelization.[16,17]

In this context, we have investigated the effects of carvacrol on wound healing induced by excisional skin injury model in rats. We have evaluated wound surface area, wound depth, and granulation of the skin in a time dependent manner. In addition, we have studied the effects of carvacrol on the expressions of pro- inflammatory- IL-1_β, TGF-β and TNF-α.

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DOI: 10.5530/pj.2014.3.2

MATERIAL AND METHOD

Animal experiments

Experiments were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the study was approved by the local ethics committee (Ankara University, Medical Faculty). In this study, 350±27 gr, male Wistar-albino rats were used in this study. Rats were equally divided into two set of experimental groups and treated with either

vehicle or carvacrol. Carvacrol was purchased by Sigma (Sigma-Aldrich; Istanbul, Turkey) and dissolwed in sunflower oil in a concentration of 12,5%. Two-hundreds microliter carvacrol containing sunflower oil was applied topically on the injured skin just after excision of the skin and repeated for 5 consecutive days. Tissue samples were collected 3 (n=10), 8 (n= 10) and 12 (n= 10) days after induction of wound injury.

The animals were anesthetized with ketamine (60 mg/kg.) and xylazine (6 mg/kg). Back, starting from the neck (2 × 3cm), of rats were completely cleaned of hair and wiped with 70% alcohol. Full-thickness skin wounds were formed on the cleaned part, as 1cm away from each of the middle line and each other, through the use of 4 mm punch biopsy tool.

Half of the tissues taken out were fixed with 10% formal solution for histological examination and the other half of the injured tissues collected were immediately frozen within liquid nitrogen for the examination of TNF-α, TGF-β_1, IL-1_β levels with enzyme linked immunosorbent assay (ELISA) method.

Pathological and histological evaluation

Photographs of scar tissues were taken by using a Canon Power shot A410 model digital camera, and surface areas of scar tissues were calculated by using the program AutoCAD (2006). For the histological examination, 5 µm tissue samples were stained with Hematoxilen-Eosin (H&E). Depth of tissue damage and thickness of granulation tissue on ulcer surface were measured by using Olympus BX50 light microscope and microscopic grid. During the evaluations, the depth between healthy epidermis on the side and the deepest area of ulcer surface was measured for determination of the depth of tissue damage. The distance between lower part of ulcer exudates on the surface and the lowest part of fibroblast was measured. In addition, the vascular proliferation and thickness of granulation tissue were evaluated.

Evaluation of TNF-α, TGF-β_1, IL-1_β levels by ELISA

Glas-Col homogenizing system with Teflon top (Glas-Col, U.S.A.) was used for homogenization of the tissue samples. 4 ml buffer solution, 200 µl PMSF and 2 µl leupeptin were respectively put into homogenization glass tube. Each two pieces of four each tissues taken out according to days were added into this mixture. Glass tube was put into another container filled with ice. Tissue was ensured to be disintegrated by means of homogenizing system with Teflon top at 3200 rpm for ten minutes. The extract obtained at the end of ten minutes was transferred into a glass centrifuge tube and centrifuged for 10 minutes at 3000xg within a centrifuge device with its ambient temperature adjusted as 4 degrees. The supernatant obtained after the centrifuge operation was transferred into polypropylene tubes and kept at -80 degrees until the time they will be used in ELISA operation.

BIOSOURCE Immunoassay Kit Rat TNF-α KRC3012 (Biosource International, Inc. USA) for TNF-α, BIOSOURCE Immunoassay Kit Rat Multispecies TGF-β_1 KAC1689 (Biosource International, Inc. USA) for TGF-β_1 and BIOSOURCE Immunoassay Kit Rat IL-1_β KRC0012 (Biosource International, Inc. USA) for IL-1_β were used throughout the study. In the preliminary study conducted for TNF-α and IL-1_β, solutions at different dilution rates were obtained from tissue homogenate solutions by using "standard dilution buffer". For TNF-α and IL-1_β, the homogenate was not diluted; for TGF-β_1, the supernatant was 10-times diluted with "standard dilution buffer".

Statistical evaluations

Variance analysis was applied in repetitive measurements for the purpose of comparing groups with regard to time. Due to the fact that main effects of group and time were found significant in consequence of variance analysis in repetitive measurements, double comparisons between group and time averages were made by examining them with Bonferroni test with confidence interval adjustment.

Parameters on the same day of carvacrol and control groups were analysed by nonparametric Mann Whitney-U test. P values <0.05 were considered statistically significant.

RESULTS

Pathological examination evaluation results

Carvacrol treatment improved wound healing processes. Scar surface measurement were conducted 3, 8 and 12 days after induction of injury. On day 12, carvacrol treatment decreased scar surface significantly (p=0.014) (Fig. 1). Histological examination showed exudate formation on day 3 in the carvacrol and vehicle treated animals (Fig. 2). Upon statistical evaluation of the data obtained from microscopic examination of scar tissue samples taken from control and carvacrol groups, wound depth on the 8th day was significantly high in carvacrol group with respect to control group (p=0.002) (Fig. 2), granulation tissue thickness on the same day was found significantly low in carvacrol group compared to control group (p=0.008) (Fig. 2).

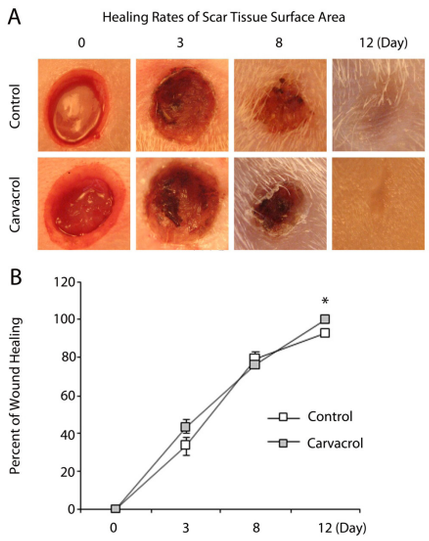

Figure 1: Macroscopic photographs of skin after excission in vehicle- and carvacrol- treated animals (A). Figure (B) shows that, healing rates of scar tissue surface area in control and carvacrol group. Healing rates of 12th day were found significantly high in carvacrol group compared to control group (on day 12, carvacrol treatment decreased scar tissue surface area significantly). The surface area of each wound tissue was measured as described in methods and reported at each time point as the percentage of the surface area at baseline.

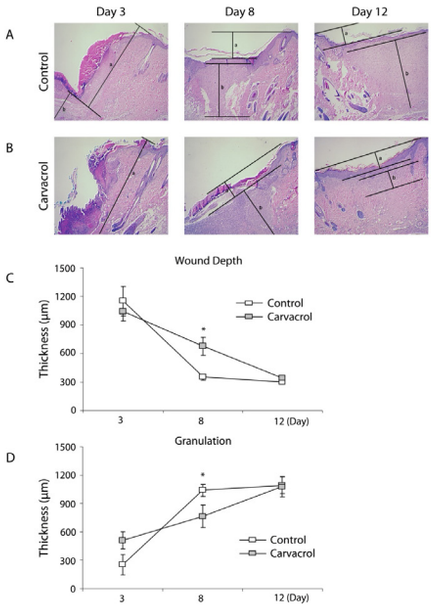

Figure 2: Microphotographs of the section of injured tissues on 3rd, 8th and 12th day after wound exposure in vehicle (A) and carvacrol (B) treated animals. Tissue samples were stained with Hematoxilen – Eosin (H& E) as described in methods. Microscopic examination of wound depth (C) and granulation (D) tissue thickness on carvacrol and control group. Wound depth on the 8th day was significantly high in carvacrol group with respect to control group (p=0.002), granulation tissue thickness on the same day was found significantly low in carvacrol group compared to control group (p=0.008).

ELISA FINDINGS

Cytokine levels of tissue homogenates obtained from scar tissues in control and carvacrol groups were measured with ELISA. TGF- β_1 levels on the 8th and 12th days were found significantly low in carvacrol group compared to control group (p=0.009) (p=0.03) (Fig. 3A). IL-1_β levels on the 8th day were found significantly high in carvacrol group compared to control group (p=0.034) (Fig. 3B).

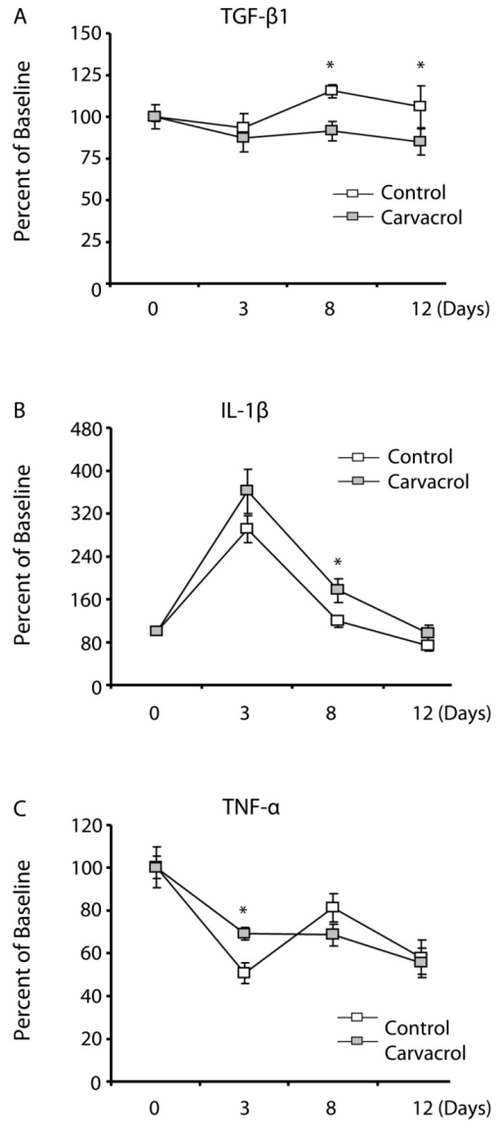

Figure 3: Level of TGF-β_1 (A), IL-1_β (B) and TNF-α (C) in vehicle and carvacrol treated animals. TGF-β_1 level was lower on the 8th and 12th days in carvacrol group compared to control group (p=0.008). IL-1_β level was significantly higher in carvacrol group compared to vehicle treated control group on the 8th day. TNF-α level was found significantly higher in carvacrol group compared to control group on day 3.
Results are expressed in percent of baseline. Statistical analysis was performed using Mann Whitney-U test. P values <0.05 were considered statistically significant.

TNF-α levels of 3rd day were found significantly high in carvacrol group compared to control group (p=0.000) (Fig. 3C).

DISCUSSION

Carvacrol is an essential oil which is present in compounds of many herbs. Apart from its known effects, its anti-microbial effects have been studied.[5,6] It inhibits prostaglandin synthesis, which plays roles in decreasing postoperative pain.[18] In this context, we have studied the roles of carvacrol in the pathophysiological events induced by excissional scin injury. First of all, we have defined the effective concentration of carvacrol. Following, we have treated animals with carvacrol at the concentration of 12.5%. Our results revealed that carvacrol- treatment improves healing rates of scar tissue surface area on the 12th day after surgery as compared with control. In contrast to increased tissue granulation, the wound depth is consonant with the condition in normal wound healing.[19] That granulation tissue thickness went up to approximately same levels on the 12th day while it was lower in carvacrol group compared to control group on the 8th day makes one think that carvacrol may affect wound healing between 8th-12th days. As a matter of fact, difference between groups in wound depth measurements on the 8th day changed in favour of carvacrol group on the 12th day, that is to say that wound depth in carvacrol group reached the same values in control group between 8th-12th days. This findings support that carvacrol may affect wound restoration process between 8th-12th days.

Different soluble factors play role in arranging and controlling phases of wound healing.[19] These factors are cytokines, growth factors, proteases, eicosanoids, quinines and cellular metabolites.[16] For this reason, it is not possible to affix events in the course of restoration process on effect of a single cytokine or growth factor.

IL-1 is a cytokine that plays a key role in inflammatory phase. It mediates synthesis and secretion of IL-1 and other proinflammatory cytokines (IL-6, G-CSF, GM-CSF) by inducing fibroblast and endothelial cells. It also leads to synthesis and secretion of more interleukin and PDGF by stimulating monocytes on wound area.[20] Leukocyte chemoattraction affects reepitalization and fibroplasia phases.[21] IL-1 level reaches to measurable levels in the first 24 hours in experimental wounds, peaks between the 1st and 3rd days and rapidly decreases through the first week.[22,23] In the study, IL-1_{β} in both of control group and carvacrol group were at measurable levels in the first day and showed a tendency to increase until the 3rd day. IL-1_{β} levels on the 8th day were significantly high in carvacrol group. This study showed that carvacrol may contribute to inflammation during the early period and reepitelization and fibroplasia processes during late period by means of increasing IL-1_{β} levels.

Besides leukocyte chemoattraction, TNF-α increases vascular proliferation and permeability.[24] TNF-α also contributes to great number of cellular metabolic events such as procurement of nutrients and acute phase protein synthesis which are essential for wound healing.[25] On the other hand, TNF-α may have negative effects on wound healing. These effects occur as inhibition in collagen synthesis depending upon decrease in productions of collagen hydroxyproline and proalpha-1 chain.[26,27] Rapala et al. has shown that TNF-α, depending on dose in acute experimental wounds, decreases granulation tissue growth on the 7th day. However, this effect was not observed on the 14th and 21st days.[28] TNF-α can be locally detected within 12 hours and, peaks after 72 hours in clinically formed wound.[29] However, our findings showed that TNF-α levels in homogenates obtained from scar tissue have a tendency to decrease until the 3rd day in control group, peaked on the 8th day and gradually decreased later on. On the contrary, TNF-α levels in carvacrol group showed a continuous tendency to decrease.

TGF-β is one of the most significant mediators in wound healing. It is secreted by T lymphocytes, endothelial cells, keratinocytes, thrombocytes in scar tissue, macrophages, smooth muscle cells and fibroblasts.[30,31] TGF-β_1, TGF-β_2 and TGF-β_3 are 3 isoforms of TGF-β found in mammals. TGF-β_1 and TGF-β_2 stimulates scar tissue formation by causing extracellular matrix deposition[32,33] via increasing extracellular matrix production and suppressing proteolysis and catabolism of extracellular matrix. On the other hand, some studies showed that TGF-β_3, unlike the other two isoforms, has activities intended to decrease scar tissue formation.[32–35] However, our results showed that TGF-β_1 expression was significantly higher in carvacrol treated animals as compared with control animals.

CONCLUSION

Here, we have evaluated the effects of carvacrol on tissue repair and time dependent expression patterns of IL-1_{β}, TGF-β_1 and TNF-α after skin injury. Our result indicated beneficial effects of carvacrol on skin injury treatment

and it was associated with a moderately increased tissue granulation in the subacute phase of injury and increased expression of TGF-β_1.

DECLARATION OF INTEREST SECTION

The authors have any financial, consulting, and personal relationships with other people or organizations that could influence (bias) the author's work.

REFERENCES

1. Baser K.H.C. Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils, Current Pharmaceutical Design, 2008; 14, 3106–3120
2. De Vicenzi M, Stammati A, De Vicenzi A, Silano M. Constituents of aromatic plants: carvacrol. Fitoterapia. 2004; 75: 801–804.
3. KırımerN,BaşerKHC,andTümenG.CarvacrolRichPlantsinTurkey. Chem. Nat. Comp. 1995; 31: 37–41.
4. Zeytinoğlu M, Aydın S, Öztürk Y, Başer KHC. Inhibitory effects of carvacrol on DMBA induced pulmonary tumorigenesis in rats. Acta Pharmaceutica Turcica 1998; 50: 93–98.
5. Ross SA, El-Keltawı NE, and Megalla SE, Antimicrobial Activity of Some Egyptian Aromatic Plants. Fitoterapia 1980; 51: 201–205
6. Thompson DP, Fungitoxic Activity Of Essential Oil Components On Food Storage Fungi. Mycologia 1989; 81: 151–153.
7. Didry N, Dubreuıı L, and Pınkas M. Antimicrobial Activity of Thymol, Carvacrol and Cinnamaldehyde Alone or in Combination, Pharmazie 1993; 48: 301–304.
8. AkgülA,KıvançM. Inhibitory Effects Of Selected Turkish Spices and Oregano Components on Some Foodborne Fungi. Int. J. Food Microbiol 1988; 6: 263–268.
9. Ali-Shtayeh MS, Al-Nuri MA, Yaghmour RMR, and Faidi YR. Antimicrobial Activity of Micromeria Nervosa From The Palestinian Area. J. Ethnopharmacol. 1997; 58: 1436–147.
10. Crippa A, and Bruno E. Antifungal Activity In Vitro of Phenols and other Natural Substances, Eco. Nat. Ecol. 1989; 7: 29–32.
11. Haraguchı H, Ishıkawa H, Sanchez Y, et al. Antioxidative Constituents in Heterotheca Inuloides, Bioorg. Med. Chem. 1997; 5: 865–871
12. Lagouri V, Boskou D. Nutrient antioxidants in oregano. Int. J. Food Sci. Nutr., 1996; 47: 493–497.
13. Cabo J, Crespo ME, Jımenez J, and Zarzuelo A. The Spasmolytic Activity of Various Aromatic Plants From The Province of Granada. I.–The Activity of The Major Components of Their Essential Oils, Plant Med. Phytother. 1986; 20: 213–218.
14. Van Den Broucke CO, and Lemlı JA. Antispasmodic Activity of Origanum compactum. Planta Med. 1980; 38: 317–331.
15. Van Den Broucke CO, and Lemlı JA. Antispasmodic Activity of Origanum compactum, Part 2, Antagonistic Effect of Thymol and Carvacrol, Planta Med. 1982; 45: 188–190.
16. Henry G, Garner WL. Inflammatory mediators in wound healing. Surg Clin N Am 2003; 83: 483–507.
17. Kondo T, and Ishida Y. Molecular pathology of wound healing. Forensic Science International 2010; 203: 93–98
18. Wagner H, Wierer M, and Bauer R. In Vitro Inhibition of Prostaglandin Biosynthesis by Essential Oils and Phenolic Compounds, Planta Med. 1986; 52: 184–187.
19. Christine L. Theoret. DMV, PhD. Diplomate ACVS; update on wound repair. Clin Tech Equine Pract. 2004; 3:110–112.
20. Herlyn M, Malkowitz S. Regulatory pathways in tumor growth and invasion. Lab Invest. 1991; 65: 262–271.
21. Sauder DN, Kilian PL, McLane JA, et al. Interleukin-1 enhances epidermal wound healing. Lymphokine Res. 1990; 9:465.
22. Fahey TJ III, Sherry B, Tracey KJ, et al. Cytokine production in a model of wound healing: The appearance of MIP-1, MIP-2, cachectin/TNF and IL-1. Cytokine 1990; 2: 92.
23. Goretsky MJ, Harriger MD, Supp AP, Greenhalgh DG, and Boyce ST. Expression of interleukin-1 alpha, interleukin-6, and basic fibroblast growth factor by cultured skin substitutes before and after grafting to full-thickness wounds in athymic mice. J. Trauma 1996; 40: 894.
24. LJ Greenfield, MW Mulholland, KT Oldham, GB Zelenock, and KD Lillemore, eds. Surgery: Scientific Principles and Practice. pp.124–147. Lippincott- Raven: Philadelphia, 1997.
25. Wilmore DW, LY Cheung, AH Harken, JW Holcroft, and JL Meakins, eds. Cytokines and the cellular response to injury and infection. Scientific American, Vol. 1: Care in the ICU. New York: Scientific American, 1996.
26. Rapala K. The effect of tumor necrosis factor alpha on wound healing: An experimental study. Ann. Chir. Gynaecol. Suppl. 1996; 211: 1.
27. Rapala K, Peltonen J, Heino J, et al. Tumor necrosis factor-alpha selectivity modulates expression of collagen genes in rat granulation tissue. Eur. J. Surg. 1997; 163: 207.
28. Rapala K, Laato M, Niinikoski J, et al. Tumor necrosis factor alpha inhibits wound healing in the rat. Eur. Surg. Res. 1991; 23: 261.
29. Feiken E, Romer J, Eriksen J, and Lund LR. Neutrophils express tumor necrosis factor-alpha during mouse skin wound healing. T. 1995; 105: 120.
30. Buckmire MA, Parquet G, Greenway S, and Rolandelli RH. Temporal expression of TGF-beta 1. EGF, and PDGF-BB in a model of colonic wound healing. J. Surg. Res. 1998; 80: 52.
31. Schultz J, Rotatori DS, and Clark W. EGF and TGF alpha in wound healing and repair. J. Cell. Biochem. 1991; 45: 346.
32. Shah M, Foreman DM, and Ferguson MWJ. Neutralising antibody to TGF-β1,2 reduces cutaneous scarring in adult rodents. Journal of Cell Science 1994; 107: 1137–1157.
33. Shah M, Foreman DM, and Ferguson MWJ. Neutralisation of TGF-β1 and TGF-β2 or exogenous addition of TGF-β3 to cutaneous rat wounds reduces scarring. Journal of Cell Science 1995; 108: 985–1002.
34. Ferguson MWJ and O'Kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention Phil. Trans. R. Soc. Lond. B 2004; 359: 839– 850.
35. Li W, Huang EY, Dudas M, et al. Transforming growth factor-β3 affects plasminogen activator inhibitor-1 expression in fetal mice and modulates fibroblast mediated collagen gel contraction. Wound Rep Reg 2006; 14: 516– 525.

Development and validation of a RP-HPLC method for the simultaneous determination of Mangiferin, Ellagic acid and Hydroxycitric acid in polyherbal formulation

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Submission Date: 12-1-2014

Accepted Date: 24-3-2014

ABSTRACT

The US patented polyherbal formulation for the prevention and management of Type II diabetes and its vascular complications was used for the present study. The formulation consists of roots of *Salacia species*, leaves of *Lagestroemia parviflora* and fruit rind of *Garcinia indica*. The use of reversed phase C_{18} HPLC column was used and eluted with isocratic mobile phase of acetonitrile and phosphoric acid buffer solution enabled the efficient separation of chemical markers within 20min. Validation of the method was performed in order to demonstrate its selectivity, accuracy, precision, repeatability and recovery. All calibration curve shows good linear correlation coefficients ($r^2 > 0.995$) within tested ranges. Three markers in this polyherbal formulation were quantified were Mangiferin (1.53% w/w), Ellagic acid (0.9655 w/w), Hydroxycitric acid (5.3% w/w). Intra and inter day RSDs of retention times and peak areas were less than 3%. The recoveries were between 95% and 102.5%. In conclusion a method has been developed for the simultaneous quantification of three markers in this polyherbal formulation. The established RP-HPLC method was simple, precise and accurate and can be used for the quality control of the raw materials as well as formulations.

Keywords: Polyherbal formulation, Mangiferin, Ellagic acid, Hydroxycitric acid, RP-HPLC

INTRODUCTION

There is always a constant and continues challenge in the standardization and quality control of the ayurvedic and polyherbal formulations in the determination of active constituents of each ingredient in the polyherbal formulation which requires optimal separation techniques of biomarkers with the high resolution and least interferences from each other. Also there is an urgent need of scientific evidence and clinical validation with chemical, biological standardization and preclinical data[1]. Recognition of the medical and health benefits of herbal medicines with health claim is growing worldwide and one of the challenges in the acceptance of herbal medicines is the lack of standardization.[2] The quality of herbal medicine, i.e., the profile of the constituents in the final product has implications in efficacy and safety, due to the complex nature of the chemical constituents of plant-based drugs. Modern analytical techniques are increasing to overcome these problems of separation, identification and determination of the active constituents for such polyherbal formulations. The advancement in the chromatographic and spectroscopic techniques made it possible to determine the active constituents in a mixture with comparatively

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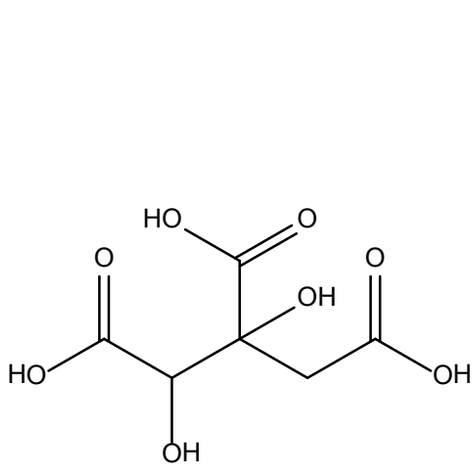
DOI: 10.5530/pj.2014.3.4

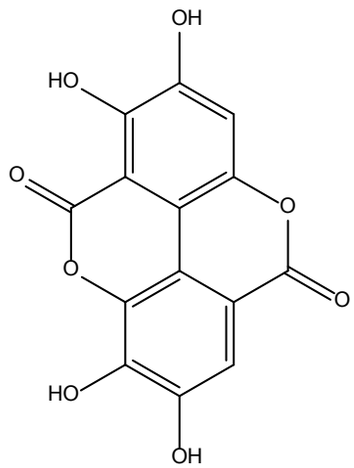
little clean-up, particularly methods using high performance liquid chromatography (HPLC) with reverse phase column are most appropriate for the analysis of multiple constituents present in the herbal preparations.

A polyherbal formulation consists of *Salacia roxbhurghii* (Hippocrateaceae), *Salacia oblonga* (Hippocrateaceae), *Garcinia indica* (Guttiferae), *Lagestroemia parviflora* (Lythraceae) for the prevention and management of Type-II Diabetes mellitus and its associated vascular complications was developed and patented by Dubey et al[3] from SRM University,TamilNadu, India. In our present investigation we have developed a simple optimized and validated RP-HPLC method for the standardization of this polyherbal formulation. Three chemical markers were selected for the quantification and one was from each medicinal herb used as raw materials, Mangiferin for Salacia species,[4] Ellagic acid for *Lagestroemia parviflora*[5](5) and Hydroxycitric acid for *Garcinia indica*.[6] The chemical structures of the compounds are depicted in Fig 1. The pharmacological actions of these markers were extensively studied and proved to have various pharmacological activities such as antimutagenic, antidiabetic,[7] antiviral, antioxidant[8] anticancer[9] weight reduction.[10]

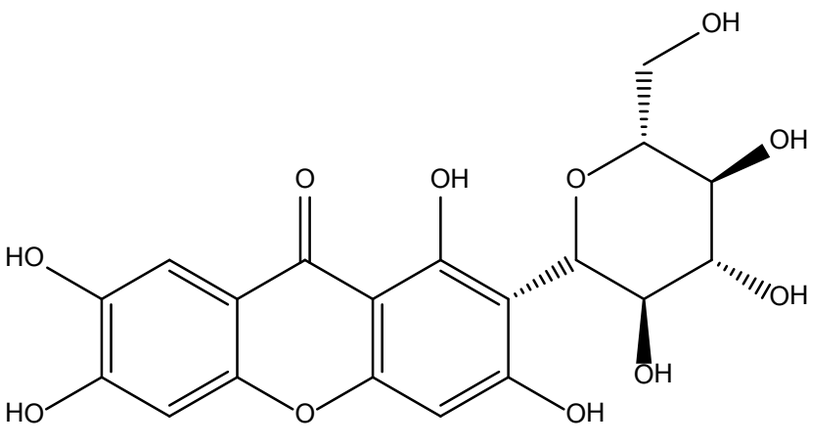
Although many approaches including thin layer chromatography[11] High performance liquid chromatography with UV-Vis.[12–14] PDA or MS/MS[15] have reported to analyze the contents of compounds mentioned above individually or collectively in two or more. However, there were no reports on simultaneous determination of these three compounds in a polyherbal formulation by RP-HPLC-PDA detection.



Hydroxycitric acid



Ellagic acid



Mangiferin

Figure 1. Chemical structures of compounds

In the present study a simple, practical and cost effective HPLC method with isocratic elution mode for the quality control of herbal formulation was proposed and successfully applied for first time. The method was validated on the basis of its sensitivity, linearity, precision, accuracy, limit of detection (LOD) and Limit of Quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines.[16] The proposed method can be used to determine the contents of Mangiferin, Ellagic acid and Hydroxycitric acid in any polyherbal formulation

MATERIALS AND METHODS

Chemicals, reagents and materials

Polyherbal formulation was purchased from M/s Varansi Bio research Pvt. Ltd. Mangiferin, Ellagic acid andHydroxycitric acid was purchased from the Sigma Aldrich (MO,USA), Chennai as a gift sample. HPLC grade Acetonitrile and methanol were obtained from Merck(Darmstadt, Germany).Water was obtained from ultra-purified Milli-Q Biocel (Millipore). All solvents and samples were filtered through MILLEX FG(Millipore), 13mm, 0.2µM, nonsterile membrane sample filter paper before injecting into system.

APPARATUS AND CHROMATOGRAPHIC CONDITIONS

The analyses were performed on a Shimadzu LC-20AD HPLC system equipped with Rheodyne 7725injection valve furnished with 20µL loop, an SPD-M20A photodiode array detector and Labsolutions software. Separation was carried out using a Phenomenex C_{18} column (250mm × 4.6mm i.d., 5µm pore size). The column was optimized and maintained at 28ºC throughout analysis and detection wavelength was set at 254nm for Mangiferin, Ellagic acid and 220nm for Hydroxycitric acid.

SAMPLE PREPARATION

The 20 intact polyherbal formulation composition capsules were weighed and powder was remained empty gelatin capsule was weighed to calculate the average weight equivalent to 100mg of *Salacia oblonga* and extracted three times with 100 mL methanol. Mixed standard stock solution was prepared by accurately (1.0mg/mL) weighing three markers i.e., Mangiferin, Ellagic acid and Hydroxycitric acid and dissolved in acetonitrile water (1:1). The working standard solution was prepared by diluting the mixed standard solution with the same to a series of proper concentrations to construct calibration curve. The standard stock and working solutions were all stored at 4ºC until use.

CALIBRATION

The contents of the markers were determined using a calibration curve established with six dilutions of each standard at concentrations ranging from 2 to 32µg/mL and each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentrations of the markers injected. The linearity was evaluated by linear regression analysis which was calculated by least square regression method. Before injecting solutions, the column was equilibrated for at least 30 min with mobile phase flowing through system. Peak identification was achieved by comparison of both the retention time (R_t) and UV absorption spectrum with those obtained for standards. The reference substances employed to construct the calibration curves were Mangiferin, Ellagic acid and Hydroxycitric acid.

VALIDATION PARAMETER

The method was validated according to ICH guidelines for linearity, precision, accuracy, selectivity, LOD and LOQ (16). Selectivity was checked using an extract of polyherbal formulation and mixture of standard solution of markers by the method in the concentration range of 2 to 32µg/mL. The accuracy of the proposed method was determined by a recovery study, carried out by adding standard markers to the extract of polyherbal formulation. The samples were spiked with three different amounts of standards prior to extraction. The spiked samples were extracted in triplicate and analyzed under the previously established optimal conditions. The obtained average contents of the target compounds were used to calculate the spiked recoveries. Precision was determined by repeatability and inter-day and intraday reproducibility experiments. A standard solution containing three markers were six times, the mean amount and standard deviation (SD) value of each constituent was calculated. The LOD and LOQ of marker compounds were calculated at signal to noise (S/N) ratio of approximately 3:1 and 10:1 respectively.

RESULTS AND DISCUSSION

Optimization of HPLC chromatographic conditions

Optimum chromatographic conditions were obtained after running different mobile phase with reversed phase C_{18} column. Many different gradient systems of mobile phases were tried to achieve the separation of peaks. Phosphate buffer solution was preferred over other mobile phases because its resulted in improved separation. Selecting 254nm for Mangiferin, Ellagic acid and 220nm for Hydroxycitric acid as the detection wavelength resulted in an acceptable response and enables the detection of all three compounds used in this study and column was maintained at 28ºC throughout analysis with flow rate of 0.5 mL/min with acetonitrile. HPLC fingerprint for polyherbal formulation was developed and elution was carried out at a flow rate of 0.5mL/min with acetonitrile as solvent A and buffer solution (0.03% v/v Phosphoric acid) as solvent B using isocratic elution at the ratio of 45:55 respectively. Each run was followed by a 10 min wash with 10% acetonitrile.

QUANTIFICATION OF MARKERS PRESENT IN POLYHERBAL FORMULATION

The three markers were found in formulation and they were quantified with respect to Mangiferin (1.56 ± 0.02) Ellagic acid (0.91 ± 0.07) and Hydroxycitric acid (5.33 ± 0.22).The chromatographs of a mixture of Mangiferin, Ellagic acid and Hydroxycitric acid and chromatogram of polyherbal formulation were shown at Fig. 2 – Fig. 8 and Polyherbal formulation showed complete separation of three markers. The results obtained are shown in Table 3.

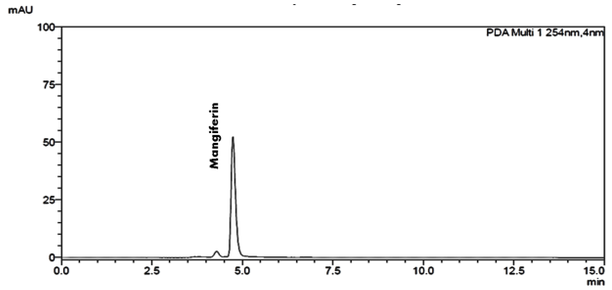


Figure 2. HPLC chromatogram of standard Mangiferin.

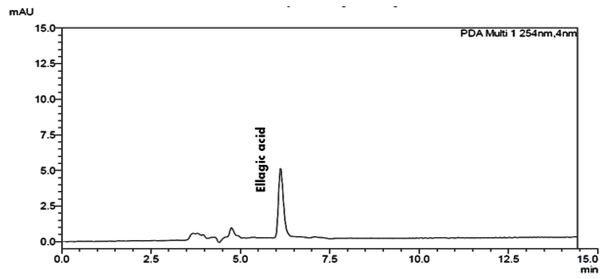


Figure 3. HPLC chromatogram of Standard Ellagic acid.

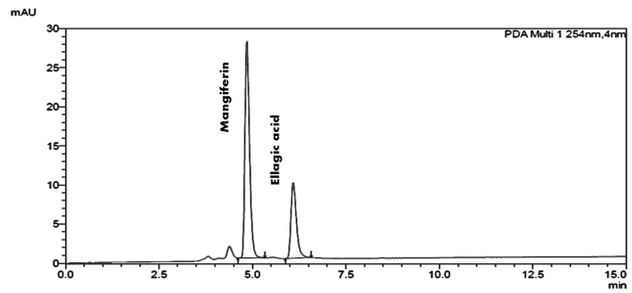


Figure 4. HPLC chromatogram of standard Hydroxycitric acid.

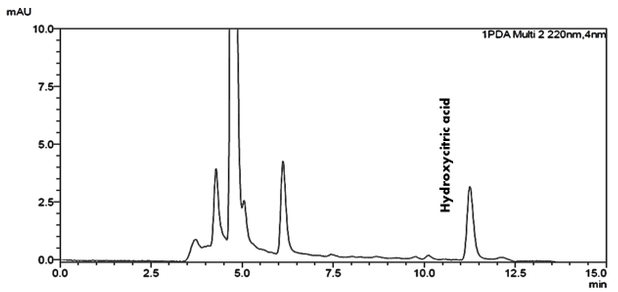


Figure 5. HPLC chromatogram of Standard mixture at 254nm.

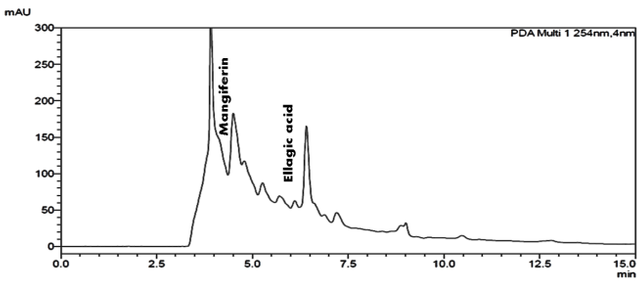


Figure 6. HPLC chromatogram of Standard mixture at 220nm.

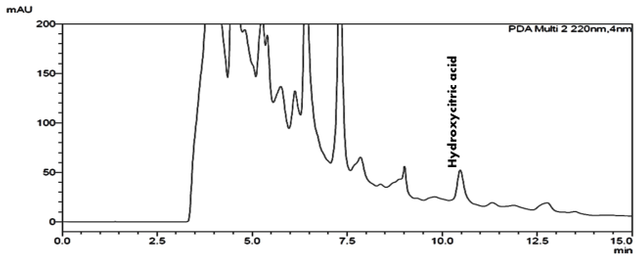


Figure 7. HPLC chromatogram of Polyherbal formulation at 254nm.

VALIDATION OF METHOD

The HPLC method was validated by defining the selectivity, linearity, accuracy, precision, LOD and LOQ. For qualitative purposes, the method was evaluated by taking into account the precision in the retention time and selectivity of marker compounds eluted. A high repeatability in the retention time was obtained for standards and extracts even at high concentration. For quantitative purpose, linearity, accuracy, precision, LOD and LOQ were evaluated. LOD and LOQ values were 2.53µg/mL and 7.66µg/mL for Mangiferin, 4.62µg/mL and 14.01µg/mL for Ellagic acid and 1.38µg/mL and 4.21µg/mL for Hydroxycitric acid respectively. Linear correlation was obtained between peak area and concentration of three markers in the range of 2 to 32µg/mL. Values of the regression coefficients (r^2) of the markers were higher than 0.99 thus confirming the linearity of the methods (Table 1), the high recovery values (95-103%) indicated a satisfactory accuracy. Relative standard deviation of all the parameters was less than 3.5% for the degree of repeatability, indicating the high repeatability of the developed method (Table 2). The low coefficient of variation values of intraday and inter day precision revealed that the method is precise (Table 4). Therefore, this HPLC method can be regarded as selective, accurate and precise.

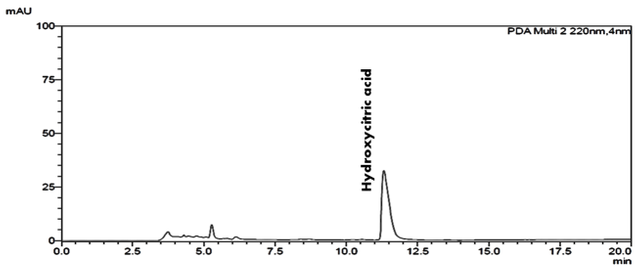


Figure 8. HPLC chromatogram of Polyherbal formulation at 220nm.

CONCLUSION

The results indicate that this polyherbal formulation contains a number of markers that may be responsible for its pharmacological activity. The developed HPLC method will assist in the standardization of this polyherbal formulation using biologically active chemical markers. The developed HPLC method for simultaneous determination of Mangiferin, Ellagic acid and Hydroxycitric acid from polyherbal formulation is accurate, precise, reproducible and repeatable, which are currently the subject of further investigation, apart from those standards studied. With growing demand for herbal drugs and increased belief in the usage of herbal medicine, the development of a standardization tool will helps in maintaining quality of herbal products

CONFLICTS OF INTEREST

All authors have none to declare

Table 1. Regression parameter, Linearity, limit of detection (LOD) and limit of quantificaiton (LOQ) of proposed HPLC method

Compound	Concentration range (µg/mL)	R_t (min)	Wavelength (nm)	Regression equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Mangiferin	2–32	4.76 ± 0.05	254	y = 31170x – 29273	0.99	2.53	7.66
Ellagic acid	2–32	6.09 ± 0.01	254	y = 7255.2x + 5704.9	0.98	4.62	14.01
Hydroxy citric acid	2–32	13.49 ± 0.11	220	y = 22592x – 9919.5	0.99	1.38	4.21

Table 2. Repeatability and recovery study for the three markers in Polyherbal formulation

Compound	Content (mg/g)	Added amount (mg)	Recorded amount(mg)	Recovery(%)	RSD(%)
Mangiferin	1.56 ± 0.02	1	2.48 ± 0.19	97.78 ± 1.43	1.46
		2	3.61 ± 0.11	101.40 ± 3.31	3.26
		4	5.83 ± 0.06	104.91 ± 1.22	1.16
Ellagic acid	0.91 ± 0.07	0.5	1.28 ± 0.07	91.67 ± 1.43	1.46
		1	1.86 ± 0.20	110.53 ± 2.95	2.66
		1.5	2.36 ± 0.05	100.00 ± 2.40	2.44
Hydroxycitric acid	5.33 ± 0.22	3	8.53 ± 0.05	102.81 ± 0.69	0.67
		6	11.66 ± 0.15	103.24 ± 1.35	1.3
		9	14.83 ± 0.30	103.72 ± 2.13	2

Table 3. Quantification of Mangiferin, Ellagic acid and Hydroxy citric acid

	Amount of Mangiferin (% w/w)	Amount of Ellagic acid (% w/w)	Amount of Hydroxy citric acid (%w/w)
Polyherbal formulation	1.56 ± 0.02	0.91 ± 0.07	5.33 ± 0.22

Table 4. Precision of the Intra-day and Inter day HPLC measurement for Mangiferin, Ellagic acid and Hydroxycitric acid in Polyherbal formulation

Compounds	Intraday		Inter-day	
	Content (%, w/w)	RSD (%)	Content (%, w/w)	RSD (%)
Mangiferin	1.51 ± 0.02	1.32	1.53 ± 0.04	2.61
Ellagic acid	0.91 ± 0.01	1.05	0.90 ± 0.02	2.22
Hydroxy citric acid	5.33 ± 0.05	0.93	5.56 ± 0.11	1.97

ACKNOWLEDGEMENTS

The authors are wish to express their gratitude to the Department of Science and Technology, Government of India, India for providing Financial assistance to carry out the research.

REFERENCES

1. Conte AA. The effects of (-)-hydroxycitrate and chromium (GTF) on obesity. Journal of American College of Nutrition. 1994;13: 535–536.
2. Dandan Han, Chen Chengjun, Zhang Cong, Zhang Yu and Tang Xing. Determination of mangiferin in rat plasma by liquid-liquid extraction with UPLC-MS/MS. Journal of pharmaceutical and biomedical analysis. 2010;51:260–263.
3. Dhalwal K, Biradar YS and Rajani M. High-performance thin-layer chromatography densitometric method for simultaneous quantitation of phyllantin, hypophyllanthin, gallic acid and ellagic acid in *Phyllanthus amarus*. Journal of AOAC international. 2006;89:619–623.
4. Dubey Govind Prasad, Aruna Agrawal, Nirupama Dubey, Shipra Dubey, R Dubey, and SM Deborah. Herbal formualtion for the prevention and management of Type-2 Diabetes mellitus and vascular complications associated with diabetes. Ed. SRM University. USA Patent 8337911B2. Dec 25, 2013.
5. Hayashi T, et al. Ellagitannins from *Lagestroemia speciosa* as activators of glucose trasport in fat cells.Planta Medica. 2002;68:173–175.
6. Igho Onakpoya, Hung Kang Shao, Perry Rachel, Wider Barbara and Ernst Edzard. The Use of Garcinia Extract (Hydroxycitric Acid) as a Weight loss supplement: A systematic Review and Meta-Analysis of Randomised Clinical Trials.Journal of Obesity 2011;1–9.
7. Ilango Kaliappan, Kammalla Kumar Ananth, Ramasamy Mohan Kumar, Agarwal Aruna, and GP Dubey. Emerging need of pharmacokinetics in Ayurvedic system of medicine. International Journal of Research in Ayurveda and Pharmacy, 2013;4:647–651.
8. International Conference on Harmonization. Guideline on Validation of Analytical Procedure-Methodology. Geneva, 1996.
9. Jayaprakasha GK, Sakariah KK. Determination of organic acids in leaves and rinds of *Garcinia indica* (Desr.) by LC. Journal of Pharmaceutical and Biomedical Analysis. 2002; 28:379–384.
10. Jayaprakasha GK, Sakariah KK. Determination of (-) Hydroxycitric acid in commerical samples of *Garcinia cambogia* extract by liquid chromatography with ultraviolet detection. Journal of Liquid chromatography and relative technology. 2000;23:915–923.
11. Khanduja KL, Gandhi RK, Pathania V and Shal N. Prevention of N-nitrosodiethylamine-induced lung tumorigenesis by ellagic acid and quercetin in mice. Food and Chemical Toxicology. 1999;37:313–318.
12. Ong ES. Extraction methods and chemical standardization of botanical and herbal preparations. Journal of Chromatography B. 2004;812:23–33.
13. Ronghua Dai, Gao Jun and Bi Kaishun. High performance liquid chromatographic method for the determination and pharmacokinetic study of mangifeirn in plasma of rats of rats having taken the traditional chinese medicinal preparation Zi-Shen Pill. Journal of Chromatographic science. 2004;42;88–90.
14. Vasudev SS, et al. Validated HPLC method for the simultaneous determination of taxol and ellagic acid in *Punica granatum* fruit extract containing combination formualtion. Pharmazie. 2012;67:834–838.
15. Yoshikawa M, Ninomiya K, Shimoda H, Nishida N, Matsuda H. Hepatoprotective and antioxidant properties of *Salacia reticulate* : Preventive effects of phenolic constituents on CCl4-induced injury in mice. Biological pharmaceutical bulletien. 2001;25:72–76.
16. Yoshikawa M, Nishida N, Shimoda H, Takada M, Kawahara Y, and Matsuda H. Polyphenol constituents from Salacia species: quantitative analysis of mangiferin with alpha-glucosidase and aldose reductase inhibitory activities.Yakugaku Zasshi. 2001;121:371–378.

Assessment of anti hyperglycemic fractions isolated from *Albizia procera* stem bark chloroform extract using STZ induced diabetic albino rats

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Submission Date: 23-1-2014

Accepted Date: 24-3-2014

ABSTRACT

Objective: The present Study was to identify more effective hypoglycemic fractions from chloroform extract of *Albizia procera* stem bark. **Material and methods:** Isolated fractions of *Albizia procera stem bark chloroform extract* were given individually to different batches of rats both normal (80 mg/kg of b.wt animals) and STZ induced diabetic rats (160mg/kg b.wt animals) after an overnight fast. The blood glucose levels were measured at 0, 1, 2, 3, 5 and 6 hours after the treatment. Fractions were also treated to STZ induced diabetic rats by chronically (80mg/kg b.wt). **Results:** The fractions E of *Albizia procera stem bark chloroform extract was* shown maximum blood glucose lowering effect in both normal and STZ diabetic rats with acute and chronic treatment. The other fractions are also showing hypoglycemic and antihyperglycemic activity, but the effect is significantly less than that of fraction E. The antihyperglycemic activity of fractions of *Albizia procera stem bark chloroform extract* was compared with the treatment of glibenclamide. **Conclusion:** The present data confirm the anti diabetic activity of *Albizia procera* in Indian traditional medicine for Diabetes mellitus treatment. The anti hyperglycemic action attributed to the presence of valuable flavonoids, terpinoids in the fraction E.

Keywords: *Albizia procera, hyperglycemia,* Streptozocin (STZ)

INTRODUCTION

Diabetes Mellitus is a chronic metabolic disease resulting from insulin deficiency and insulin resistence.[1] It is a complex disorder in the way the body's ability to convert food into energy is impaired. Consequently, glucose builds up in the blood instead of moving into cells, called hyperglycemia, if left untreated, hyperglycemia can become severe and contribute to the development of microvascular and macrovascular complications of diabetes.[2] In the long term, persistent hyperglycemia, even if not severe, can lead to complications affecting your eyes, kidneys, nerves and heart. However, Diabetes care is complex and requires that many issues addressed, beyond glycemic control.[3]

Treatment of diabetes mellitus by insulin and oral hypoglycemic drugs fails to prevent diabetes related complications in many patients, indicating that additional alternative treatments could be helpful.[4] Herbal medicines have been used in medical practice for thousands of years and are recognized especially as a valuable and readily available healthcare resource. During the past decades, the contribution of herbal medicines and their preparations has attracted much interest in the pharmaceutical industry. However, most herbal medicines still need to be investigated scientifically, although the experience obtained from their traditional use over the years should not be ignored.[5, 14]

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DOI: 10.5530/pj.2014.3.5

Albizia procera is a medium-sized, fast growing, pioneer tree species belonging to the Mimosaceae. It also occurs in tropical semi-evergreen, moist deciduous and northern subtropical forests.[6] All parts of the plant are reported to show anticancerous activity. The plant is used for stomach & intestinal disease and during pregnancy; bark decoction is given for rheumatism and hemorrhages.[7] Arial parts of extracts reported α glucosidase inhibitory activity, which was useful to prevent postprandial elevated glucose level in type II diabetes mellitus.[8] The aim of present study is to evaluate the hypoglycemic and anti hyperglycemic activities of fractions isolated from chloroform extract of *Albizia procera* stem bark.

MATERIALS AND METHODS

Collection and processing of plant material

About 2 kg of the stem bark sheaths of *Albizia procera* were collected from the deciduous forest of Thirumala in Andhra Pradesh State, India, in the months of March and June 2013. Samples were authenticated by Dr. Madhave setti, Department of Botany, Sri Venkateswara University. The stem bark sheaths were cleaned and dried in hot air oven under 50°C for 2 days. These were ground to powder using the laboratory Hammer mill. Powdered samples were collected and stored in air- and water-proof containers protected from direct sunlight and heat until required for extraction.

Preparation of extract

Obtained powder (1Kg) was macerated with different solvents Petroleum ether, chloroform, ethanol and distilled water at room temperature for 7 days and obtained yield was 1.45g/kg, 2.9 g/kg and 315g/kg respectively.

Isolation process of fractions

Thin-layer chromatography method was carried out using silica gel aluminum plate 60F-254, 0.5mm (TLC plates, Merck). The solvent system used for TLC was Petroleum ether/ethyl acetate (7:3). The spots were visualized in UV light and 10% of H_2SO_4 in methanol. The chloroform extract was subjected to column chromatography (silica gel 60-100) for further purification. The column was equilibrated for one hour with petroleum ether at flow rate 5 ml/min. The sample was (1 g dissolve in chloroform) loaded on to the column, fourteen fractions were collected using Petroleum ether, Petroleum ether: Chloroform (9:1), Petroleum ether: Chloroform (7:3), Chloroform: Petroleum ether (9:1), Chloroform: methanol (9.5:0.5), Chloroform: methanol (9:1), Chloroform: Methanol (8:2). Above yielded product were pooled into six fractions based on TLC. The yield values of fraction A 72.5mg/g, fraction B 102.5 mg/g, fraction C 115 mg/g, fraction D 125mg/g, fraction E (107.5mg/g) and fraction F 227.5mg/g, respectively. All six fractions were checked for their hypoglycemic and anti hyperglycemic activity.

Phytochemical analysis

Phytochemical analysis of fractions was carried out by different methods.[9]

Experimental design

Ethics statement

All animal experiments were conducted with the approval of the Institutional Animal Care Committee and Committee for purpose conducting supervising experimental animals (IACC& 439/PO/01/a/CPCSEA) of Shri Vishnu college of Pharmacy, Bhimavaram, Andhra University, India.

Animals

Albino rats of Wistar strain weighing 150-200g were purchased from MKM, Hyderabad. The rats were kept in polypropylene cages (3 in each cage) at an ambient temperature of 25±2°C and relative humidity of 55–65%. A 12 h light and dark schedule was maintained in the air conditioned animal house. All the rats were fed with common diets for 1 week after arrival, and then divided into groups with free access to food and water.

Evaluation of hypoglycaemic effect of isolated fraction from Albizia procera stem bark of chloroform extract on normoglycemic rats[10]

Male Wistar normoglycemic rats (150–200gm) were used in the experiment. All experiments were carried out using six animals per group.

Groups	Treatment
I	Normal group (Tween 80)
II	Normoglycemic rats + Glibenclamide (10 mg/kg)
III	Normoglycemic rats + Fraction A (80 mg/kg)
IV	Normoglycemic rats + Fraction B (80 mg/kg)
V	Normoglycemic rats + Fraction C (80 mg/kg)
VI	Normoglycemic rats + Fraction D (80 mg/kg)
VII	Normoglycemic rats + Fraction E (80 mg/kg)
VIII	Normoglycemic rats + Fraction F (80 mg/kg)

Blood samples were collected from the tail vein at 0, 1, 2, 3, 4, 5 and 6 hr after oral administration. Blood glucose concentration was estimated by enzymatic glucose oxidase method using a commercial glucometer (Accue check active). The percentage variation of glycemia for each group was calculated in relation to initial (0 h) level, according to:

% glycemic change = G0 – Gt × 100/Go

Where G0 were initial glycemia values and Gx were the

STZ induced diabetic rats[10]

Diabetes was induced in male albino rats by intraperitoneal administration of STZ (a single dose 45 mg/kg b.wt) dissolved in freshly prepared 0.01 M citrate buffer (p^h 4.5). After 72 hrs rats with marked hyperglycemia (blood glucose ≥ 300 mg.dl) were selected and use for study.

Design of Anti hyperglycaemic effect of isolated fraction from Albizia procera stem bark of chloroform extract by acute treatment on STZ induced diabetic rats

Male Wistar normoglycemic rats (150–200 g) were used in the experiment. All experiments were carried out using six animals per group.

Group	I	Normal group (Tween 80)
Group	II	Diabetic rats
Group	III	Diabetic rats + Glibenclamide (20 mg/kg)
Group	IV	Diabetic rats + Fraction A (160 mg/kg)
Group	V	Diabetic rats + Fraction B (160 mg/kg)
Group	VI	Diabetic rats + Fraction C (160 mg/kg)
Group	VII	Diabetic rats + Fraction D (160 mg/kg)
Group	VIII	Diabetic rats + Fraction E (160 mg/kg)
Group	IX	Diabetic rats + Fraction F (160 mg/kg)

Blood samples were collected from the tail vein at 0, 1, 3, 4, 5 and 6 hr after treatment administration. Blood glucose concentration was estimated by enzymatic glucose oxidase method using a commercial glucometer (Accue check active) The percentage variation of glycemia for each group was calculated in relation to initial (0 h) level, according to:

% glycemic change = G0 – Gt × 100/Go

Where G0 were initial glycemia values and Gx were the final glycemia.

Design of Anti hyperglycaemic effect of isolated fraction from Albizia procera stem bark of chloroform extract by chronic treatment on STZ induced diabetic rats

Male Wistar normoglycemic rats (150–200 gm) were used in the experiment. All experiments were carried out using six animals per group.

Group	I	Normal group (Tween 80)
Group	II	Diabetic rats + Tween 80
Group	III	Diabetic rats + Glibenclamide (10 mg/kg)
Group	IV	Diabetic rats + Fraction A (80 mg/kg)
Group	V	Diabetic rats + Fraction B (80 mg/kg)
Group	VI	Diabetic rats + Fraction C (80 mg/kg)
Group	VII	Diabetic rats + Fraction D (80 mg/kg)
Group	VIII	Diabetic rats + Fraction E (80 mg/kg)
Group	IX	Diabetic rats + Fraction F (80 mg/kg)

Diabetic rats were treated with isolated fraction for 14 days, blood glucose concentrations were determined by Coralyzer 100. Percentage glycemic variation was calculated as a function of time (t) by using formula:

% glycemic change = G0 – Gt × 100/Go

Go and Gt represent glycemic value before (i.e., Zero time and glycemic value at 7days after administration of the extracts, respectively).

ANALYSIS

Results were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using Graph pad prism with one-way analysis of variance (ANOVA). A difference was defined as significant when $P<0.05$.

RESULTS

Effect of various fractions on the blood glucose level of normal rats.

Table 2, (Fig 1) shows the effect of isolated fractions on the fasting blood glucose level in normoglycemic rats. Among these, fraction D and E showed significant hypoglycemic activity 60.4%, 67.3% respectively. 53.1% and 51.2% of glucose reduction with fraction B and C, 44.01% and 36.7% of glucose reduction with fraction F and A with 80 mg/kg b.wt. after 6 hr treatment in normoglycemic rats.

Table 1. Phytochemical analysis

Tests	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E	Fraction F
Alkaloids	–	–	–	–	–	–
Terpenoids	+	+	+	+	+	+
Saponins	–	+	–	+	–	–
Cardiac glycosides	–	+	–	–	+	–
Tanins	–	–	–	–	–	–
Carbohydrates	+	–	–	+	–	+
Proteins	–	–	–	–	–	–
Flavanoids	+	–	–	–	+	+

Table 2. Effect of different fractions isolated from *Albizia procera* stem bark chloroform extract on normoglycemic rats

Groups	Blood glucose levels at different time intervals (hrs)							% of glucose reduction
	0 hr	1 hr	2hr	3hr	4hr	5 hr	6hr	
I	123 ± 2.3	120.5 ± 1.9	119 ± 2.1	112 ± 1.5	110 ± 1.3	106 ± 1.4	109 ± 1.1	11.3
II	124.7 ± 2.1	72.5 ± 3.5	43.4 ± 1.4	48.3 ± 1.3	58.4 ± 2.1	64.2 ± 2.3	60.4 ± 3.2	51.5
III	131.6 ± 2.2	147.2 ± 2.5	103.3 ± 1.3	83.2 ± 1.4	85.4 ± 1.3	87.5 ± 1.8	92.2 ± 2.1	36.7
IV	116.3 ± 1.2	92.3 ± 1.6	86 ± 1.4	54.5 ± 1.3	69 ± 1.4	73.4 ± 2.3	81.3 ± 1.4	53.1
V	128.3 ± 2.4	102.3 ± 1.3	62.6 ± 1.4	70.3 ± 1.4	79.4 ± 2.3	72.6 ± 1.3	66.4 ± 1.9	51.2
VI	107.3 ± 1.3	91.3 ± 2.1	101.8 ± 2.5	86.3 ± 1.2	82.4 ± 1.2	42.4 ± 1.3	47.8 ± 1.3	60.4
VII	120.4 ± 2.3	109.5 ± 1.4	71.3 ± 1.2	60 ± 2	62.23 ± 1.2	39.3 ± 1.1	48.6 ± 1,2	67.3
VIII	90.2 ± 1.3	86 ± 2	70.4 ± 1.3	61.3 ± 1.4	64.2 ± 1.3	50.5 ± 1.4	64 ± 1.8	44.01

Values are given as mean ± SEM. Values were significant P < 0.05.

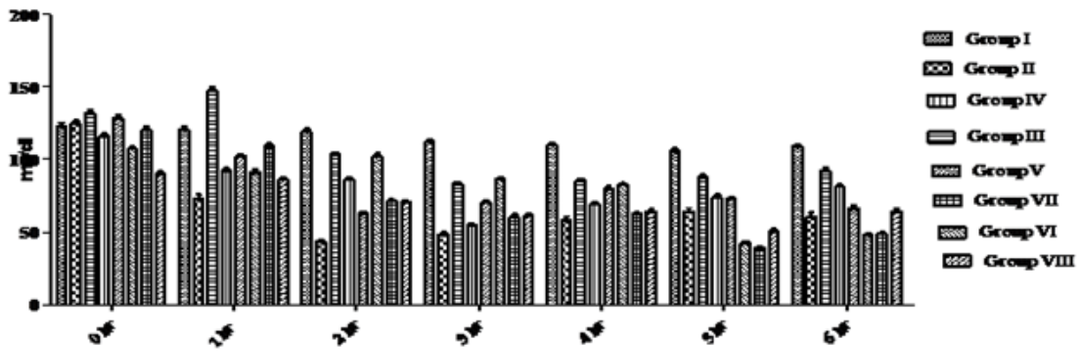

Figure 1. Effect of different fractions isolated from *Albizia procera* stem bark chloroform extract on normoglycemic rats.

Effect of acute treatment of different fractions isolated from Albizia procera stem bark chloroform extract on STZ induced diabetic rats.

Table 3, (Fig 2) shows, 41%, 49.3% and 42% of glucose reduction with fractions A, B, C (dose 160 mg/kg of body weight) at 3rd hr after oral administration in diabetic rats. Similarly, at 6th hr 76.4%, 81.02%, 51% of reduction of blood glucose levels in diabetic rats was observed with fraction D, E and F at 160 mg/kg b.wt. Treatment of glibenclamide at a dosage of 20 mg/kg.b.wt. Diabetic rats resulted in 91.3% of fall of blood glucose after 5 hrs.

Effect of chronic treatment of different fractions isolated from Albizia procera stem bark chloroform extract on STZ induced diabetic rats

The effects of isolated fractions on blood glucose in diabetic rats with chronic treatment are shown in Table 4, Fig 3. A significant decrease in blood glucose levels was

Table 3. Effect of acute treatment of different fractions isolated from *Albizia procera* stem bark chloroform extract on STZ induced diabetic rats

Groups	Blood glucose level (mg/dl)							% of reduced Glucose
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
I	128 ± 3	120 ± 2	123 ± 1.5	120 ± 2.2	121 ± 2.4	115 ± 3.3	123 ± 4.2	10.1
II	358 ± 14	340 ± 13	342 ± 21	355 ± 16	341 ± 13	331 ± 12	353 ± 16	7.5
III	435 ± 13	321 ± 21.3	159 ± 13.4	53 ± 11.3	39 ± 4.1	46 ± 2.3	69 ± 3	91.03
IV	394 ± 12	342 ± 16	283 ± 12	232 ± 14	335 ± 11	361 ± 12	369 ± 10	41
V	384.6 ± 13	378.3 ± 17	352.4 ± 13	194 ± 9	342 ± 15	369 ± 14	398 ± 13	49.3
VI	392 ± 14	314.2 ± 12	252 ± 13	227.2 ± 13	289 ± 16	301 ± 12	329 ± 13	42
VII	492.7 ± 23	445 ± 21	386 ± 17	329 ± 15	291.5 ± 14	230 ± 12	121 ± 6	76.04
VIII	325.5 ± 14	281 ± 13	200 ± 12	170.5 ± 12	121.75 ± 5	104.7 ± 6	61.7 ± 4	81.03
IX	340.5 ± 18	370 ± 15	370.2 ± 13	369 ± 14	313 ± 18	261 ± 13	166.8 ± 9	51

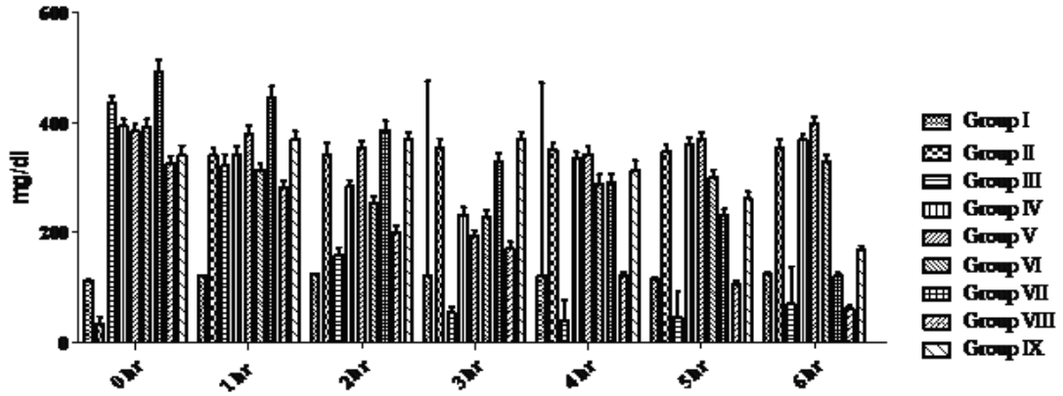

Figure 2. Effect of acute treatment of different fractions isolated from *Albizia procera* stem bark chloroform extract on STZ induced diabetic rats.

Table 3. Effect of acute treatment of different fractions isolated from *Albizia procera* stem bark chloroform extract on STZ induced diabetic rats

Groups	Blood glucose level (mg/dl)							% of reduced Glucose
	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	
I	128 ± 3	120 ± 2	123 ± 1.5	120 ± 2.2	121 ± 2.4	115 ± 3.3	123 ± 4.2	10.1
II	358 ± 14	340 ± 13	342 ± 21	355 ± 16	341 ± 13	331 ± 12	353 ± 16	7.5
III	435 ± 13	321 ± 21.3	159 ± 13.4	53 ± 11.3	39 ± 4.1	46 ± 2.3	69 ± 3	91.03
IV	394 ± 12	342 ± 16	283 ± 12	232 ± 14	335 ± 11	361 ± 12	369 ± 10	41
V	384.6 ± 13	378.3 ± 17	352.4 ± 13	194 ± 9	342 ± 15	369 ± 14	398 ± 13	49.3
VI	392 ± 14	314.2 ± 12	252 ± 13	227.2 ± 13	289 ± 16	301 ± 12	329 ± 13	42
VII	492.7 ± 23	445 ± 21	386 ± 17	329 ± 15	291.5 ± 14	230 ± 12	121 ± 6	76.04
VIII	325.5 ± 14	281 ± 13	200 ± 12	170.5 ± 12	121.75 ± 5	104.7 ± 6	61.7 ± 4	81.03
IX	340.5 ± 18	370 ± 15	370.2 ± 13	369 ± 14	313 ± 18	261 ± 13	166.8 ± 9	51

observed in diabetic treated group from initial range 398.7± 19 mg/dl to the level of 119.6 ± 9 mg/dl with 70% glucose reduction after 14 days treatment of fraction E at dose 80 mg/kg b.wt. At the dose of 10 mg/kg b.wt. glibenclamide shows initial 512 ± 25 mg/dl to the level of 152.6 ± 13 mg/dl with 70.1% after 14 days treatment.

Table 4. Effect of chronic treatment of different fractions isolated from *Albizia procera* stem bark chloroform extract on STZ induced diabetic rats

Groups	Blood glucose levels (mg/dl)		% of glucose reduction
	Before treatment	After treatment	
I	123 ± 12	112.2 ± 15	8
II	395.14 ± 13	376.5 ± 15	4.7
III	512 ± 15	152.6 ± 13	70.1
IV	382.3 ± 13	224.8 ± 12	41.1
V	423.8 ± 12	201.3 ± 15	52.5
VI	398.4 ± 14	183.4 ± 13	53.9
VII	382.5 ± 13	139.3 ± 10	63.5
VIII	398.7 ± 19	119.6 ± 9	70
IX	387.4 ± 13	211.9 ± 14	45.3

Values are given as mean ± SEM. Values were significant $P < 0.05$.

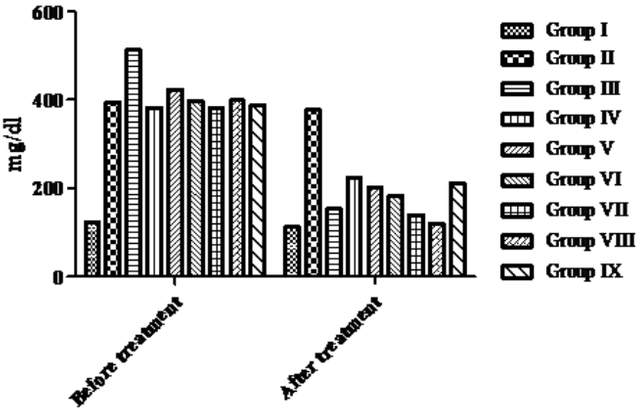

Figure 3. Effect of chronic treatment of different fractions isolated from *Albizia procera* stem bark chloroform extract on STZ induced diabetic rats.

DISCUSSION

In this study STZ was selected for induction of diabetes in rats rather than alloxan. STZ is well known for its selectively pancreatic beta- cell cytotoxicity and has been extensively used to induce diabetes mellitus in animals[11] and it is less toxic than alloxan and allows a consistent maintenance of diabetes mellitus. The present results on the normal rats indicate that the fraction E induces a significant and pronounced hypoglycemic effect (Table 2, Fig 1). The light elevation of glycemia observed in rats treated with fraction A, due to the carbohydrates contained in these fractions. This study also reported fraction E produced maximum anti hyperglycemic activity in acute treatment (81.02%) and chronic treatment (70%) in diabetic rats (Table 3 and 4, Fig 2 and 3). However fraction E, like glibenclamide produced significant reduction in blood glucose in both normal and STZ treated diabetic rats. It indicates the hypoglycemic effect of fraction E would appear to be most probably exerted via mechanism similar to that of glibenclamide. However, the possibility exists that the fraction's mimic or improve insulin's action at the cellular levels.

The data obtained in the present study do not allow any definite conclusion to be drawn on the mechanisms of action of fractions in the experimental paradigms used. However, phytochemical screening of isolated fractions from *Albizia procera* stem bark revealed that the presence of traces of cardiac glycosides, terpinoids and flavonoids. A number of investigators reported that flavonoids, terpinoids posses hypoglycemic properties in various experimental models.[12, 13] The fraction E which has shown the maximum anti hyperglycemic action was considered as the active fraction from above isolated fractions due to presence of valuable phytochemicals.

The results of present study clearly indicate that fraction E isolated from *Albizia procera* stem bark chloroform extract posses the anti hyperglycemic principles. However, chemical and pharmacological investigations are necessary to identify the latter and confirm its mechanism of action and anti diabetic potentials.

ACKNOWLEDGEMENT

We are grateful to the Dr.D.Basavaraju, Professor, Department of Pharmaceutical sciences, Shri Vishnu College of Pharmacy, Bhimavaram for his encouragement. The authors are thankful to the Shri Vishnu College of Pharmacy, for its financial support.

REFERENCES

1. Ross SA, Gulve EA, Wang M, Chemistry and biochemistry of type 2 diabetes. Chemical Review. 2004; 104:1255–1282.
2. Taskinen MR Diabetic dyslipidemia. Atherosclerosis Supplements. 2002; 3:47–51.
3. American Diabetes Association. Standards of Medical Care in Diabetes. 2011; 34:11.
4. Cherng JY, Shih MF, Potential hypoglycemic effects of Chlorella in streptozotocin-induced diabetic mice. Life Science. 2005; 77: 980–990.
5. Jacobson MF, Silverglade B, Editor – Functional foods: health boon or quackery? British Medical Journal 1999; 319(24): 205-206.
6. Asolker LV, Kakkar KK, Chakre OJ, Supplement to glossary of Indian medicinal plants. Part-I (A-K), National Institute of Science Communication. 2000:116–7.
7. Kirthikar KR, .Basu DB, Indian Medicinal Plants. Vol. 4.2n ed. Dehradun: Oriental Enterprises 2000; 7:1255–1257.
8. Tanasorn TR, Anusorn and Nijsiri R, α-glucosidase inhibitory activity of thai mimosaceous plant extracts, J Health Res. 2008; 22: 29–33.
9. Harbourne JB, Phytochemical Methods: A Guide to Modern Technique of Plant Analysis, 2nd ed. Chapman & Hall, London 1984; 282–286.

10. Hamdan II and Afifi FU, Studies on the in vitro and in vivo hypoglycemic activities of some medicinal plants used in treatment of diabetes in Jordanian traditional medicine. Journal of Ethnopharmacology. 2004; 9: 117-121.
11. Raju K, Balaraman, Anti diabetic mechanisms of saponins *Momordica cymbalaria.* Phcog Mag. 2008; 4 (15).
12. Marles and Farnsworth, 1995. Anti diabetic plants and their active constituents. Phytomedicine. 1995; 2: 137-189.
13. Akah and Okafor, 1992. Blood sugar lowering effects of *Vernonia amygdalina* in experimental rabbit model. Phytotherapy Research. 1992; 6: 171-173.
14. Taylor JLS, Rabe T, McGaw LJ, Jager AK, Staden VJ, Towards the scientific validation of traditional medicinal plants. Plant Growth Regulation. 2001; 34; 1: 23-37.

Chemical Constituents of *Dendrobium williamsonii*

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Submission Date: 30-1-2014

Accepted Date: 24-3-2014

ABSTRACT

Objective: Isolation of compounds from *Dendrobium williamsonii* and evaluation of each isolates for its free radical scavenging, antiherpetic and cytotoxic activities. **Results:** Six phenolic compounds were isolated including tetratriacontanyl-*trans*-*p*-coumarate (1), *trans*-docosanoylferulate (2), 3,3′-dihydroxy-4,5-dimethoxybibenzyl (3), moscatilin (4), apigenin (5) and vanillic acid (6). Among these isolates, compounds 3, 4 and 5 exhibited recognizable DPPH free radical scavenging potential.Only 3 exhibited weak activity against *Herpes simplex* virus, whereas 3 and 4 showed cytotoxicity against KB and MCF-7 cancer cells. **Conclusion:** This study is the first report on the chemical and biological properties of *D. williamsonii.*Compounds 3, 4 and 5 are responsible for free radical scavenging activity of this plant. Compound 4 showed the strongest cytotoxic effect on KB cancer cells.

Keywords: *Dendrobium williamsonii,* Orchidaceae, Anti-herpetic Cytotoxicity, Free radical scavenging activity

INTRODUCTION

The genus *Dendrobium* (Orchidaceae) is represented by more than 1,100 species widely distributed throughout Asia and Australia, and in Thailand about 150 species of *Dendrobium* have been identified.[1] The stems of several *Dendrobium* species have been used in traditional Chinese medicine as tonics to reduce fever and promote the production of body fluid.[2] They are also used to treat kidney and lung disorders, stomach diseases, red tongue, swelling, dry mouth, hyperglycemia and diabetes.[3] Plants of the genus *Dendrobium* (Orchidaceae) have been known to produce a wide variety of chemical compounds, including alkaloids, bibenzyls, phenanthrenes, fluorenones, steroids, sesquiterpenes, coumarins and polysaccharides.[4,5] Recent biological studies have shown that some *Dendrobium* species possess anti-platelet aggregation, anti-fibrotic, free radical scavenging, immunomodulatory and cytotoxic activities.[5–8]

Dendrobium williamsonii Day &Rchb. f. is known in Thai as "Ueangngoen sad".[9] It is also known as Williamson's *Dendrobium*. This plant species is found in Thailand, India, Vietnam, China and Mianmar.[9] In Yunnan Province of China, the decoction of stems or whole plant from *D. williamsonii*has been used as poultice to treat adynamia, dyspepsia, numbness of limbs, and injuries from falls and fracture.[10] In Thailand, this plant is an ornamental plant without recorded traditional medicinal uses.Prior to this investigation, there were no reports on the chemical and biological properties of this plant. As a part of our continuing studies on bioactive phenolics from *Dendrobium* plants[11–14], an extract prepared from the whole part of this plant was evaluated and found to possess DPPH radical scavenging, cytotoxic, and anti-herpetic activities. In this communication, we report the chemical components of this plant, as well as our studies on their DPPH free radical scavenging, cytotoxic and anti-herpetic properties.

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DOI: 10.5530/pj.2014.3.6

MATERIALS AND METHODS

General experimental procedures

Mass spectra were recorded on a Micromass LCT mass spectrometer (ESI-MS). NMR spectra were recorded on a BrukerAvance DPX-300 FT-NMR spectrometer or a Varian Unity INOVA-500 NMR spectrometer. Microtiter plate reading was performed on a Perkin-Elmer Victor™ 1420 multilabel counter.Vaccuum-liquid chromatography (VLC) and column chromatography (CC) were performed on silica gel 60 (Merck, Kieselgel 60, 70-320 mesh) and silica gel 60 (Merck, Kieselgel 60, 230-400 mesh), respectively. Size-exclusion chromatography was conducted on Sephadex LH-20 (25-100 μm, Pharmacia Fine Chemical Co. Ltd.).

Plant material

The whole plant of *D. williamsonii* Day &Rchb. f. was purchased from Jatujak market, in July 2010. Plant identification was done by Prof. ThatreePhadungcharoen (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand). A voucher specimen (BS-DW-072553) is on deposit at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Extraction and Isolation

Dried and powdered whole plant of *D. williamsonii* (1.8 kg) was extracted with MeOH(3 × 10 L) at room temperature to give a viscous mass of dried extract (165 g) after evaporation of the solvent. This material was initially subjected to vacuum-liquid chromatography (VLC) on silica gel (*n*-hexane-EtOAc, gradient) to give 6 fractions (A-F). Fraction D (9.1 g) was further separated by column chromatography (CC) over silica gel, eluted with *n*-hexane-EtOAc (gradient) to give 10 fractions (D1-D10). Fraction D3 (650 mg) was subjected to CC (silica gel; *n*-hexane-$CHCl_2$, gradient) to give 33 fractions. Compound 1(3 mg) was obtained from fraction 26 after recrystallization from *n*-hexane. Fraction 16 was purified on Sephadex LH-20 (CH_2Cl_2-MeOH, 1:1) to furnish 2 (38 mg). Fraction D5 (790 mg) was separated by CC (silica gel; *n*-hexane-EtOAc, gradient) and then further purified on Sephadex LH-20 (CH_2Cl_2-MeOH, 1:1) to furnish 3 (20 mg). Fraction D6 (1.7 g) was subjected to repeated CC (silica gel; *n*-hexane-EtOAc, gradient) to give 7 fractions (D6.1-D6.7). Fraction D6.5 (30 mg) was separated on Sephadex LH-20 (CH_2Cl_2-MeOH, 1:1) to yield 4 (5 mg). Compound 5 (5 mg) was obtained from fraction D6.6 after purification on Sephadex LH-20 (CH_2Cl_2-MeOH, 1:1). Fraction D-7 (863 mg) was separated by CC (silica gel; CH_2Cl_2-acetone, gradient) to give 13 fractions. Separation of fraction 3 (50 mg) was performed by CC over silica gel (CH_2Cl_2-MeOH, gradient) to give 6 (3 mg).

Tetratriacontanyl-*trans-p*-coumarate (1)

White powder, $C_{43}H_{76}O_3$, ESI-MS *m/z* 663 $[M+Na]^+$.^{1}H NMR (500 MHz, $CDCl_3$) δ:0.85(3H, t, *J* = 7.0 Hz, CH_3), 1.23 (62H, br s, CH_2-n), 1.67 (2H, m, CH_2-2′), 4.16 (2H, t, *J* = 7.0 Hz, CH_2O-1′), 6.27 (1H, d, *J* = 16.0 Hz, H-*β*), 6.82 (2H, d, *J* = 8.5 Hz, H-3, H-5), 7.40 (2H, d, *J* = 8.5 Hz, H-2, H-6), 7.60 (1H, d, *J* = 16.0 Hz, H-α). ^{13}C NMR (125 MHz, $CDCl_3$) δ:14.1 (CH_3), 22.6-31.9 (long chain CH_2), 64.7 (CH_2O-1′), 115.5 (C-*β*), 115.8 (C-3, C-5), 127.1 (C-1), 129.9 (C-2, C-6), 144.4 (C-α), 157.8 (C-4), 167.7 (OC=O).

trans-Docosanoylferulate (2)

White powder, $C_{32}H_{54}O_4$, ESI-MS *m/z* 525 $[M+Na]^+$.^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.85 (3H, t, *J* = 7.0 Hz, CH_3), 1.23 (38H, br s, CH_2-n), 1.67 (2H, m, CH_2-2′), 3.90 (3H, s, MeO-3), 4.16 (2H, t, *J* = 7.0 Hz, CH_2O-1′), 6.26 (1H, d, *J* = 16.0 Hz, H-*β*), 6.89 (1H, d, *J* = 8.0 Hz, H-5), 7.01 (1H, d, *J* = 1.5 Hz, H-2), 7.05 (1H, dd, *J* = 8.0, 1.5 Hz, H-6), 7.58 (1H, d, *J* = 16.0 Hz, H-α). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1 (CH_3), 22.7-31.9 (long chain CH_2), 55.9 (MeO-3), 64.6 (CH_2O-1′), 109.3 (C-2), 114.7 (C-5), 115.6 (C-*β*), 123.0 (C-6), 127.0 (C-1), 144.6 (C-α), 146.7 (C-3), 147.9 (C-4), 167.4 (OC=O).

Dihydroxy-4,5-dimethoxybibenzyl (3)

Brown amorphous solid.$C_{16}H_{18}O_4$, ESI-MS*m/z* 275 $[M+H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.83 (4H, br s, H_2-α ,H_2- α′), 3.83 (3H, s, MeO-2), 3.89 (3H, s, MeO-4), 6.27 (1H, brs, H-6), 6.49 (1H, brs, H-2), 6.68 (1H, brs, H-2′), 6.76 (1H, brd, *J* = 7.6, H-6′), 7.15 (1H, brd, *J* = 7.5, H-4′), 7.16 (1H, dd, *J* = 7.6 ,7.5 Hz, H-5′).^{13}C NMR (75 MHz, $CDCl_3$) δ:37.5 (C-α), 37.6 (C- α′), 55.8,60.9 (MeO-3, MeO-4),104.6 (C-6), 108.0 (C-2), 112.9 (C-4′), 115.4 (C-2-2′), 120.7 (C-6′), 129.4 (C-5′), 133 (C-1′), 138.1 (C-4), 143.5 ((C-1), 148.9 (C-3), 152.1 (C-5), 155.7 (C-3′).

Moscatilin (4)

Brown amorphous solid.$C_{17}H_{20}O_5$ ESI-MS*m/z* 305 $[M+H]^+$.^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.80 (4H, br s, H_2-α ,H_2-α′), 3.83 (9H, s, MeO-3, MeO-3′, MeO-5′), 6.34 (2H,s, H-2,6), 6.60 (1H, brs, H-2′), 6.66 (1H, brd, *J* = 8.1 Hz, H-6′), 6.82 (1H, d, *J* = 8.1 Hz, H-5′).^{13}C NMR (75 MHz, $CDCl_3$)δ: 37.8(C-α), 38.4 (C- α′), 55.2 (MeO-3′),

55.8 (MeO-3, MeO-5) 105.2 (C-2,6), 111.2 (C-2′), 114.1 (C-5′), 121.0(C-6′), 132.8 (C-1), 132.9 (C-1′), 133. (C-4), 143.7 (C-4′), 146.2 (C-3′), 146.8(C-3,5).

Apigenin (5)

Yellow powder, $C_{15}H_{10}O_5$ ESI-MS*m/z*271 $[M+H]^+$. ^1H NMR (300 MHz, Acetone -d_6) δ: 6.24 (1H,d, *J* = 1.7 Hz, H-6), 6.54 (1H, d, *J* = 1.7 Hz, H-8), 6.63 (1H, s, H-3), 7.01 (2H, d, *J* = 8.7 Hz, H-3′,5′), 7.95 (2H, d, *J* = 8.7 Hz, H-2′,6′), 13.01 (1H,br s, HO-5). ^{13}C NMR (75 MHz, Acetone -d_6)δ: 94.7 (C-8), 99.7 (C-6), 104.1(C-3), 105.3 (C-10) , 116.9 (C-3′,5′), 123.3 (C-1′), 129.2 (C-2′,6′), 158.8 (C-9), 161.9 (C-4′), 163.4 (C-5), 164.9 (C-7), 165.1 (C-2), 183.1 (C-4).

Vanillic acid (6)

Colorless powder, $C_8H_8O_4$,ESI-MS *m/z* 169 $[M+H]^+$.^1H NMR (500 MHz, Acetone -d_6) δ: 3.89 (3H, s, MeO-3), 6.89 (1H, d, *J* = 8.5 Hz, H-5), 7.55 (1H, d, *J* = 2.0 Hz, H-2), 7.58 (1H, dd, *J* = 8.5, 2.0 Hz, H-6). ^{13}C NMR (125 MHz, Acetone-d_6) δ: 56.3 (MeO-3), 113.4 (C-2), 115.5 (C-5), 122.9 (C-1), 124.8 (C-6), 148.0 (C-4), 152.0 (C-3), 167.4 (COOH).

DPPH radical scavenging method

The free radical scavenging effect of the samples was assessed by measuring their ability to decolor a methanolic solution of 1, 1,-diphenyl-2-picrylhydrazyl radical (DPPH, Sigma).[11] Briefly, test samples were initially prepared as a solution in EtOH (1000 μg/ml). Each compound was first tested at the concentration of 100 μg/ml. An IC_{50} value was determined if the compound showed more than 50% inhibition. For IC_{50} analysis, two fold serial dilutions were performed to give seven concentrations. The test was done by addition of the sample solution (20 μl) to the solution of 50 μM DPPH in EtOH (180 μl) in a 96-well microtiter plate. The reaction mixture was incubated at room temperature for 30 min, and then its absorbance at 510 nm was measured with a microplate reader. Quercetin (Sigma) and vitamin C were used as positive control.

Assay of Anti-HSV activity

Antiviral activity against HSV-1 (Strain KOS) and HSV-2 (Strain 186) was determined using plaque reduction method, as previously described.[15] Briefly, virus (30 PFU/25 ml) was mixed with 25 ml of complete medium containing various concentrations of test compound and then incubated at 37ºC for 1h. After incubation, the mixtures were added to Vero cells (6 × 10^5 cells/well) in 96-well microtiter plates and incubated at 37ºC for 2 h. The overlay medium containing the various concentrations of test compound was added to the Vero cells and incubated at 37ºC in humidified CO_2 incubator for 2 days. Then, virus growth inhibition was evaluated by counting the virus plaque forming on Vero cells compared with the controls. The cells also were stained with 1% crystal violet in 10% formalin for 1 h. The percent plaque inhibition was determined. Acyclovir was used as positive control.

Cytotoxic activity assay

Evaluations of cytotoxic activity against KB (epidermoid carcinoma of oral cavity) and MCF-7 (breast cancer) cancer cells were performed by using resazurinmicroplate assay (REMA).[13]Ellipticine, doxorubicin and tamoxifen were used as positive controls, and 0.5% DMSO was used as a negative control.Briefly, cells at a logarithmic growth phase were harvested and diluted to 7 × 10^4 cells/ml for KB and 9 × 10^4cells/ml for MCF-7 in fresh medium. Consecutively, 5 ml of test sample diluted in 5% DMSO, and 45 ml of cell suspension were added to 384-well plates then incubated at 37ºC in 5% CO_2 incubator. After incubation for 3 days, 12.5 ml of 62.5 mg/ml resazurin solution was added to each well, then incubated at 37ºC for 4 hours. After that the fluorescent signal at the excitation and emission wavelengths of 530 nm and 590 nm, respectively, were measured with a SpectraMax M5multi-detection microplate reader (Molecular Devices, USA). The percent inhibition of cell growth was calculated by using the following equation.

$$\% \text{ Inhibition} = [1-(FU_T/FU_C)] \times 100$$

Whereas, FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. The dose response curves were plotted from 6 concentrations of 2-fold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) can be acquired by using the SOFTMax Pro software (Molecular Devices, USA).

RESULTS AND DISCUSSION

Phytochemical investigation of the MeOH extract of the whole plant of *D. williamsonii* led to the isolation of six phenolic compounds (Figure 1). The structures of the isolates were determined through analysis of their spectroscopic data in comparison with reported values, and were identified as tetratria contanyl-*trans*-*p*-coumarate(1)[16,17], *trans*-docosanoylferulate(2)[18], 3,3′-dihydroxy-4,5-dimethoxybibenzyl (3)[19],moscatilin (4)[20], apigenin (5)[21] and vanillic acid (6)[22].

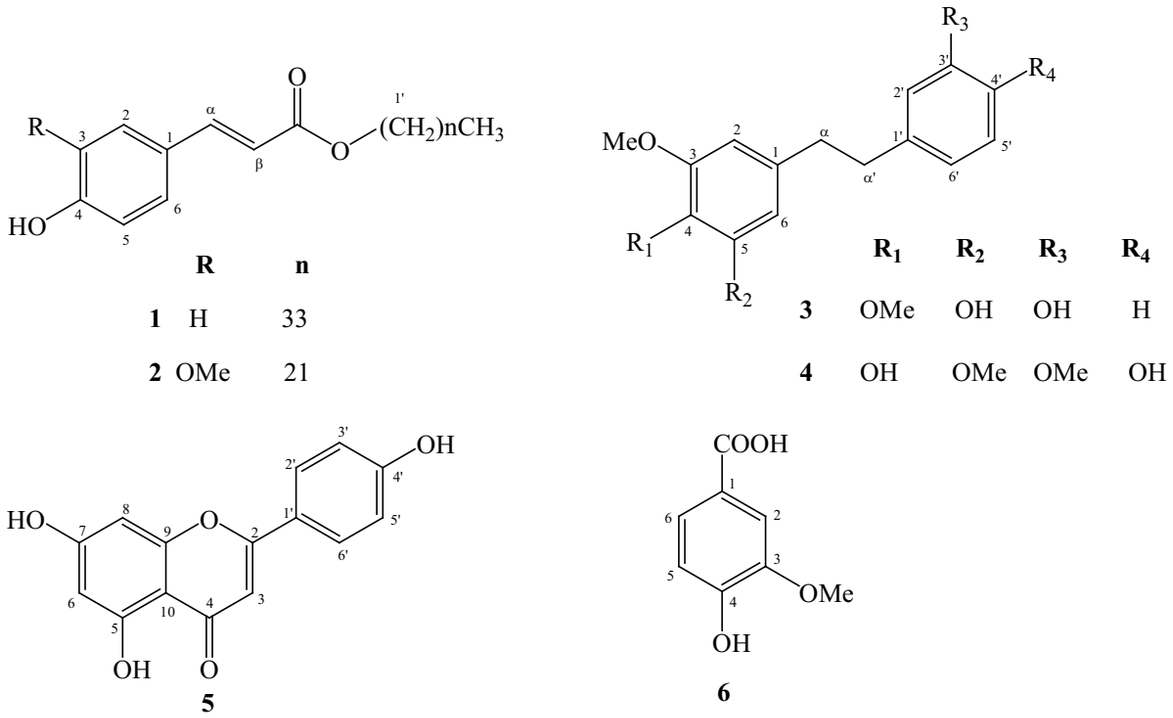

Figure 1. Compounds 1-6 isolated from *D. williamsonii*.

To the best of our knowledge, this is the first report on the chemical constituents and biological activities of *D. williamsonii*. Among the isolated compounds (1–6), tetratriacontanyl-*trans-p*-coumarate (1) and 3, 3′-dihydroxy-4, 5-dimethoxybibenzyl (3) were identified from *Dendrobium* and Orchidaceous plants for the first time. Compounds 1 and 3 were the first isolated from the root sprouts of *Agrimoniapilosa* (Rosaceae)[16] and the leaves of crowberry (*Empetrumnigrum*, Ericaceae)[19], respectively, and there are no records of biological activity of both compounds.

These isolates (1–6) were then evaluated for DPPH free radical scavenging, cytotoxic and anti-HSV activities and the results are shown in Table 1. For the DPPH radical scavenging activity, moscatilin (4) showed the strongest activity with IC_{50} 8.5 μM, whereas 3,3′-dihydroxy-4, 5-dimethoxybibenzyl (3) and apigenin (5) exhibited appreciable activity (IC_{50} 19.5 and 19.3 μM, respectively), as compared with the positive controls quercetin and vitamin C (IC_{50} 8.3 and 42.4 μM, respectively). Apigenin, a flavonoid found in many plants such as Chinese cabbage, garlic, guava, and celery, was found to possess strong radical scavenging against several reactive oxygen species.[23,24] Moreover, this compound can inhibit lipid peroxidation in isolated rat hepatocytes.[25] Free radical scavenging of moscatilin also have been report.[4] Recent study demonstrated that moscatilin was able to inhibit human lung cancer cell migration and invasion via an attenuation of endogenous reactive oxygen species especially suppression of hydroxyl radical.[26] For cytotoxic activity, as expected, moscatilin (4), a compound previously identified from several other *Dendrobium* species[27], showed strong cytotoxic activity against KB cell line with an IC_{50} value of 2.6 μM but exhibited weak activity against MCF-7 cell line (IC_{50}112.4 μM), in comparison with the positive controls ellipticine (IC_{50} 1.8 μM) and doxorubicin (IC_{50} 1.0 μM). Therefore, it should be noted that moscatilin (4) has a high selectivity to KB cell line. In addition, 3, 3′-dihydroxy-4, 5-dimethoxybibenzyl (3) exhibited weak cytotoxicity against KB cells(IC_{50} 195.0 μM) and MCF-7 cells (IC_{50} 187.7μM). Previously, cytotoxicity of moscatilin against several cancer cell lines has been reported.[14] Moscatilin was able to induce apoptosis in colorectal cancer cells through tubulin depolymerization and DNA damage stress.[28] This compound also exhibited anti-angiogenic activity by inhibition of angiogenic factor signaling pathway.[29] As above data, moscatilin has been shown to be a potential anticancer agent, and *D. williamsonii* can be asource for this compound. For anti-HSV activity, only compound 3 exhibited antiviral effect against HSV-1 and HSV-2 (IC_{50} 304.1 μM and 334.5 μM, respectively), when compared with acyclovir (IC_{50} 1.5 μM and 2.9 μM, respectively), whereas, other compounds were devoid of anti-herpetic activity.

Table 1. IC_{50} values (μM) for DPPH free radical scavenging, cytotoxic and anti-herpetic activities

Compound	DPPH	Cytotoxicity (IC_{50}, μM)		IC_{50} (μM)	
	IC_{50} (μM)	KB	MCF-7	HSV-1	HSV-2
1	NA	NA	NA	NA	NA
2	NA	NA	NA	NA	NA
3	19.5	195.0	187.7	304.1	334.5
4	8.5	2.6	112.4	NA	NA
5	19.3	NA	NA	NA	NA
6	NA	NA	NA	NA	NA
Ellipticine	NA	1.8	NA	NA	NA
Doxorubicin	NA	1.0	15.1	NA	NA
Tamoxifen	NA	NA	24.9	NA	NA
Quercetin	8.3	NA	NA	NA	NA
Vitamin C	42.4	NA	NA	NA	NA
Acyclovir	NA	NA	NA	1.5	2.9

NA = less than 50 % inhibition at 100 μg/mL.

Conflicts of interest

All authors have none to declare.

ACKNOWLEDGMENTS

This work was supported by research grants from Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University, and from The Thailand Research Fund (BRG 5580004). We thank Prof. Thatree Phadungcharoen and Prof. Vimolmas Lipipun (Chulalongkorn University) for the plant identification, and the assays of antiherpetic activity, respectively. Thanks are also due to The Research Instrument Center of the Faculty of Pharmaceutical Sciences, for providing research facilities.

REFERENCES

1. Seidenfaden G, Orchid genera in Thailand XII. *Dendrobium* Sw.*Opera Bot*. 1985; 83:1–295.
2. Hu J, Fan W, Dong F, Miao Z, Zhou J, Chemical components of *Dendrobium chrysotoxum*.*Chin. J. Chem*. 2012; 30:1327–1330.
3. Hossain MM, Therapeutic orchids: traditional uses and recent advances-an overview. *Fitoterapia* 2011; 82:102–140.
4. Zhang X, Xu JK, Wang J, Wang NL, Kurihara H, Kitanaka S, Yao XS, Bioactive bibenzyl derivatives and fluorenones from *Dendrobium nobile*.*J. Nat. Prod*. 2007; 70:24–28.
5. Ito M, Matsuzaki K, Wang J, Daikonya A, Wang NL, Yao XS, Kitanaka S, New phenanthrenes and stilbenes from *Dendrobium loddigesii*.*Chem. Pharm. Bull*. 2010; 58:628–633.
6. Hu JM, Chen JJ, Yu H, Zhao YX, Zhou J, Two novel bibenzyls from *Dendrobium trigonopus*.*J. Asian Nat. Prod.Res*. 2008;10:647–651.
7. Yang H, Sung SH, Kim YC, Antifibrotic phenanthrenes of *Dendrobium nobile* stems. *J. Nat. Prod*. 2007; 70:1925–1929.
8. Lo SF, Mulabagal V, Chen CL, Kuo CL, Tsay HS, Bioguided fractionation and isolation of free radical scavenging component from in vitro propagated Chinese medicinal plants *Dendrobium tosaense* Makino and *Dendrobium moniliforme* SW.*J. Agric. Food Chem.* 2004; 52:6916–6919.
9. Vaddhanaphuti N, *A field guide to the wild orchids of Thailand Forth and expanded edition*. Silkworm Books, Chiang Mai, 2005, p. 132.
10. Long CL, Li R, Ethnobotanical studies on medicinal plants used by the Red-headed Yao people in Jinping, Yunnan Province, China. *J. Ethnopharmacol*. 2004; 90:389–395.
11. Sritularak B, Anuwat M, Likhitwitayawuid K, A new phenanthrene quinone from *Dendrobium draconis*.*J. Asian Nat. Prod. Res.* 2011; 13:251–255.
12. Sritularak B, Likitwitayawuid K, New bisbibenzyls from *Dendrobium falconeri*.*Helv.Chim.Acta* 2009; 92:740–744.
13. Phechrmeekha T, Sritularak B, Likhitwitayawuid K, New phenolic compounds from *Dendrobium capillipes* and *Dendrobium secundum*.*J. Asian Nat. Prod. Res.* 2010; 14:748—754.
14. Chanvorachote P, Kowitdamrong A, Ruanghirun T, Sritularak B, Mungmee C, Likhitwitayawuid K, Anti-metastatic activities of bibenzyls from *Dendrobium pulchellum*. *Nat. Prod. Commun*. 2013; 8:115–118.
15. Sritularak B, Tantrakarnsakul K, Lipipun V, Likhitwitayawuid K, Flavonoids with anti-HSV activity from the root bark of *Artocarpus lakoocha*. *Nat. Prod. Commun*. 2013; 8:1079–1080.
16. Pei YH, Li X, Zhu TR, Studies on the structure of a new isocoumarin glucoside of the root sprouts of *Agrimoniapilosa* Ledeb.*Yaoxue Xuebao* 1989; 24:837–840.
17. Mahmood U, Kaul VK, Acharya R, Jirovetz L. *p*-Coumaric acid esters from *Tanacetum longifolium*. *Phytochemistry* 2003; 64:851–853.
18. Ulubelen A, Topcu G, Olçal S, Rearranged abietane diterpenes from *Teucrium divaricatum* subsp. *Villosum*. *Phytochemistry* 1994; 37:1371–1375.
19. Arriaga-Giner FJ, Wollenweber E, Dörr M, Bibenzyls from crowberry leaves, *Phytochemistry.*1993; 33:725–726.
20. Majumder PL, Sen RC, Moscatilin, a bibenzyl derivative from the orchid *Dendrobium moscatum*. *Phytochemistry.*1987; 26: 2121–2124.
21. Han XH, Hong SS, Hwang JS, Lee MK, Hwang BY, Ro JS, Monoamine oxidase inhibitory components from *Cayratia japonica*.*Arch. Pharm. Res*.2007; 30:13–17.
22. Sakushima A, Coşkun M, Maoka T, Hydroxybenzoic acids from *Boreavaorientalis*. *Phytochemistry* 1995; 40:257–261.

23. Miean KH, Mohamed S, Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J. Agric. Food Chem.* 2001; 49:3106–3112.
24. Ellnain-Wojtaszek M, Kruczyński Z, Kasprzak J, Investigation of the free radical scavenging activity of *Ginkgo biloba* L. leaves. *Fitoterapia* 2003; 74:1–6.
25. Nuutila AM, Puupponen-Pimiä R, Aarni M, Oksman-Caldentey KM, Comparison of antioxidant activities of onion and garlic extracts by inhibition of lipid peroxidation and radical scavenging activity. *Food Chem.* 2003; 81:485–-493.
26. Kowitdamrong A, Chanvorachote P, Sritularak B, Pongrakhananon V, Moscatilin inhibits lung cancer cell motility and invasion via suppression of endogenous reactive oxygen species. *BioMed Res. Inter.* 2013;Article ID 765894:1–11.
27. Ho CK, Chen CC, Moscatilin from the orchid *Dendrobium loddigesii* is a potent anticancer agent, *Cancer Invest*. 2003; 21:729–736.
28. Chen TH, Pan SL, Guh JH, Liao CH, Huang DY, Chen CC, Teng CM, Moscatilin induces apoptosis in human colorectal cancer cells: a crucial role of c-Jun NH_2-terminal protein kinase activation causes by tubulin depolymerization and DNA damage. *Clin. Cancer Res*. 2008; 14:4250–4257.
29. Tsai AC, Pan SL, Liao CH, Guh JH, Wang SW, Sun HL, Liu YN, Chen CC, Shen CC, Chang YL, Teng CM, Moscatilin, a bibenzyl derivative from the India orchid *Dendrobium loddigesii*, suppress tumor angiogenesis and growth *in vitro* and *in vivo*. *Cancer Lett.* 2010; 292:163–170.

Chromatographic studies on *Benincasa hispida* (thunb.) Cogn. Seed extract scrutinized by HPLC and HPTLC

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ABSTRACT

Background: *Benincasa hispida* (Thunb.) Cogn. is an extensive climbing annual herb in an agricultural country like India. Lupeol, a constituent of this species, has been reported to possess good amount of pharmacological potential. Objective: In the current studies, the research team focused on determining the percentage of the lupeol present in the extract of *Benincasa hispida* seeds by chromatographic techniques. **Materials and Methods:** Shade-dried seeds of *Benincasa hispida* were subjected to soxhlet extraction followed by scrutinization of the lupeol contents by HPTLC and HPLC methods after carrying out preliminary phytochemical screening for the constituents present in the extract. **Results:** The extraction yield was found to be 1.2% (w/w). Phytochemical screening of the extract revealed the presence of carbohydrates, glycosides, alkaloids, fixed oils and fats, tannins phenolic compounds, steroids and flavonoids. The amount of lupeol present in the seeds extract was found to be 0.47% w/w (HPTLC)and 6.85% w/v (HPLC) by HPTLC quantification and HPLC analysis respectively. Research studies showed a peak which coincided with the peak of standard lupeol signifying the presence of lupeol in the extract. **Conclusion:** The extract contains significant amount of lupeol.

Keywords: *Benincasa hispida,* Soxhlet extraction, HPLC, HPTLC, lupeol

INTRODUCTION

India has one of the richest medicinal plant traditions in the world with remarkable contemporary relevance for ensuring health security to millions. Around 25,000 effective plant-based formulations and folk medicines are known to rural communities that are used for medicinal purposes in preventive and curative applications.[1] We have selected *Benincasa hispida* (Thunb.) Cogn. species for our research studies which is found to be cultivated throughout the plains of India, Burma and Ceylon on hills upto 4000ft. In addition, it is described as best fruit among all *Valliphala*. The classical medicines reported from the selected plant are Kushmanda avaleha, Vasakhanda, Khanda and Rasayana.[2–4] Due to its rasayana property, it is beneficial in improving immune protection and is advised during the degenerative phase of life around 45 years in both the sexes.[5] The fruit contains good amount of proteins, enzymes, vitamin B_1 and C, flavonoid C-glycoside, terpenes, phenolic acids and free sugars such as glucose, rhamnose, mannitol, uronic acid and some trace metals which are beneficial in treatment of various diseases such as diabetes, cancer, inflammatory disorders, convulsions and infections. Pectic polysaccharides have been obtained from the fruits by sequential extraction[6,7] while three phenolic compounds *viz*. astilbin, catechin and naringenin by high-speed counter current chromatography.[8] Bioactive proteins were isolated and characterized from each of the parts and the highest yield was reported in roots.[9]

Taking into considerations the literature citations, our research article attempts to find out how much percentage of the lupeol is present in the *Benincasa hispida* seeds extract which is reported for having a number of important bioactivities such as antiarthritic, antiproto-

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DOI: 10.5530/pj.2014.3.7

zal, anti-inflammatory, anticancer, hepatoprotective and chemoprevention.[10,11] Though literature reports several methods for quantification of lupeol from various plants, no studies on bioactivity guided fractionation for isolation and quantification of lupeol from *Benincasa hispida* seeds extract has been reported.

MATERIALS AND METHODS

Part A: Collection, authentication and extraction

Fresh seeds of *Benincasa hispida* were collected from Mumbai local market in the month of April-May and shade-dried. They were authenticated by Agharkar Research Institute, Pune. A voucher specimen (No.3/187/2013/Adm.1692/083) was deposited in the botany department of Agharkar Research Institute, Pune. The seeds were subjected to a soxhlet extraction procedure as follows:

Step 1: 31 gm of seed powder was packed in soxhlet thimble.
Step 2: 300 ml petroleum ether was taken in round bottom flask.
Step 3: The sample was extracted until the solvent in soxhlet thimble become colorless.
Step 4: The concentrate was evaporated to dryness under reduced pressure at 40°C using rotary evaporator.
Step 5: The extract was collected and stored in an airtight amber colored glass container.

This petroleum ether extract of *Benincasa hispida* seeds was subjected to analytical studies by comparison with standard biomarkers after carrying out the preliminary qualitative phytochemical screening.[12–14] All the standard biomarkers used for identification purpose in analytical studies were obtained from Sigma-Aldrich Private Limited, India, and solvents from Merck, India, including HPLC grades. Separation and identification of active components from the seed extract formed the basis for HPTLC and HPLC methods. These are sophisticated powerful visualization techniques which are preferred in the detection of the constituents in the extracts due to its accuracy, preciseness, specificity, sensitivity and reproducibility.[15]

Part B: Analytical studies

Our research studies encompasses Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High Pressure Liquid Chromatography (HPLC) for determining the percentage of lupeol present as an active constituent in the selected seed extract of BH plant species.

(a) Thin Layer Chromatography (TLC)

Mobile Phase: Toluene: Ethyl Acetate (9.5:0.5) was used for the study. The standard and the sample were dissolved in ethanol and was filtered using Whatman Filter paper no. 41. The TLC chamber was saturated for 30 mins.

(b) High Performance Thin Layer Chromatography (HPTLC)

The HPTLC was performed at Radiant Research Laboratories Private Limited, Bangalore. The analysis was carried out by application of the sample and the standard dissolved in methanol on HPTLC plates (20 × 10 cm) coated with silica gel 60 F254. Scanning of the developed plates was carried out at 333nm and 550nm. The standard and the sample were prepared by dissolving 5.16mg and 47.5mg in 10ml of solvent each. Spots of 3 µg/l, 6 µg/l,9 µg/l and 12 µg/l were applied on HPTLC plates. Instrument used was CAMAG Linomat 5 with spray gas as an inert gas, sample solvent as methanol, dosage speed of 150 nl/s and predosage volume: 0.2 µl. The HPTLC details comprises of syringe size of 100 µl. Ten tracks with application position of 12.0 mm and band length of 8.0 mm were used. Calibration mode of single level, statistics mode with CV and evaluation with peak height and area (percentage) were the analyzed parameters.

Formula: Percentage of lupeol = sample area × standard dilution × purity × 100/standard area × sample dilution × 100

(c) High Pressure Liquid Chromatography (HPLC) analysis

HPLC instrument used was Shimadzu LC-10 ATVP with software as Chromtech N 2000 data with a detector of 280 nm and a flow rate of 1.5 ml/min. The injection volume was 20µl and column dimensions were RP C-18, 250 × 4.6 mm, 5 µ. Mobile phase used was acetonitrile and water (95:5). 100µg of both, the standard and sample were dissolved in 1 ml of the solvent. From this stock solution, 20 µl was injected for experimental purpose.

Formula: Percentage of lupeol = sample area × standard dilution × purity × 100/standard area × sample dilution × 100.

RESULTS

(a) Extraction yield

The extraction yield was found to be 1.2% w/w (petroleum ether extract).

(b) Preliminary analysis of the plant extracts

The petroleum ether extract was been found to be positive for carbohydrates, glycosides, alkaloids, fixed oils and fats, tannins, phenolic compounds, steroids and flavonoids. (Table 1).

(c) Chromatographic analysis of extracts

(i) TLC reports

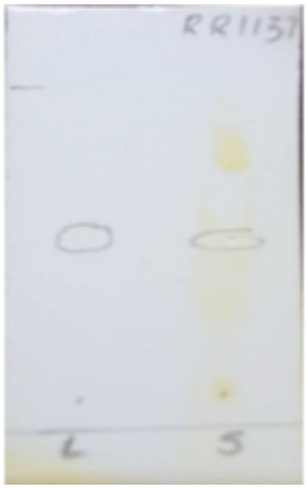
Rf value for standard lupeol and the extract was found to be 0.21 which confirms the presence of the active constituent in the seed extract (Figure 1).

(ii) HPTLC reports

The seed extract showed well resolved spots at tracks 5 and 6 in comparison to the standard at tracks 1, 2, 3, 4, 9, 10, 11 and 12. The images were obtained at 333 nm and 550 nm before and after derivatization respectively. The Rf value was found to be equal with lupeol [(Start 0.50, maximum 0.56 and end 0.57)] (Table 2 and Figures 2–11). The amount of lupeol present in the extract was **0.47 % w/w** (*i.e.* 0.22 mg of lupeol present in 47.5 mg of extract).

Table 1. Preliminary qualitative phytochemical analysis of the plant extract

Test for	Reagent	Observation
Carbohydrates	Molish's reagent	Present
Reducing sugars	Fehling's reagent	Present
	Benedict's reagent	Present
Saponin glycosides	Formation of Foam	Present
Flavonoids	Shinoda reagent	Present
Alkaloids	Dragendorff's reagent	Present
	Hager's reagent	Present
	Wagner's reagent	Present
	Mayer's reagent	Present
Tannins and Phenolic compounds	5% FeCl3 solution	Present
	Bromine water	Present
	Dilute iodine solution	Present
Mucilage with powdered drug material	Ruthenium red	Absent
	Swelling property	Absent
Steroids	Salkowski reagent	Present
	Liebermann-Burchard reagent	Present
	Lieberman reagent	Present
Fats and Oils	Sudan Red III reagent	Present
	Filter paper	Present
	Saponification	Present



Mobile Phase: Toluene: Ethyl Acetate (9.5:0.5)
L=Lupeol (Extract) S = Lupeol (Standard)

Figure 1. TLC of Benincasa hispida.

Table 2. HPTLC analysis of Benincasa hispida and standard lupeol

Track No	Details	Height	Area
1	lupeol (3µg/l)	146.3	4123.5
2	lupeol (6µg/l)	265.6	7144.5
3	lupeol (9µg/l)	332.6	9307.5
4	lupeol (12µg/l)	384.1	11146.7
5	*Benincasa hispida* petroleum ether extract (6 µg/l)	12.4	330.3
6	*Benincasa hispida* petroleum ether extract (9 µg/l)	125.1	3061.5
9	lupeol (12µg/l)	370.9	10527.7
10	lupeol (9µg/l)	313.2	8656.6
11	lupeol (6 µg/l)	239.2	6272.9
12	lupeol (3µg/l)	130.6	3500.4

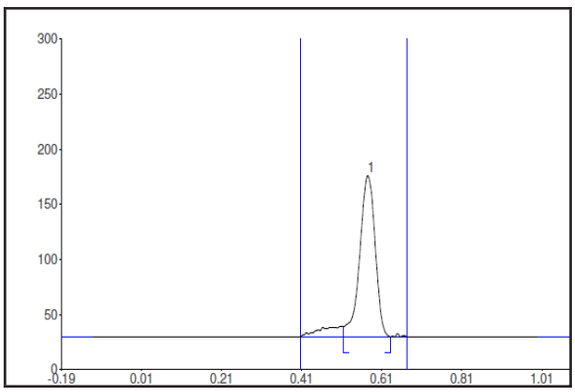


Figure 2. Track 1 - HPTLC peak of standard Lupeol.

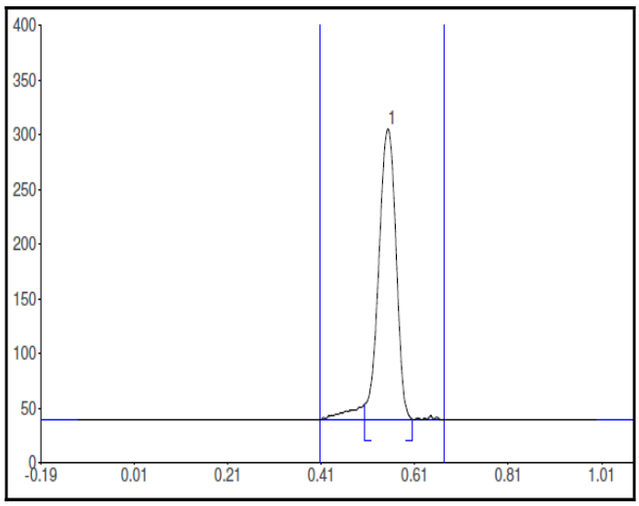


Figure 3. Track 2 - HPTLC peak of standard lupeol.

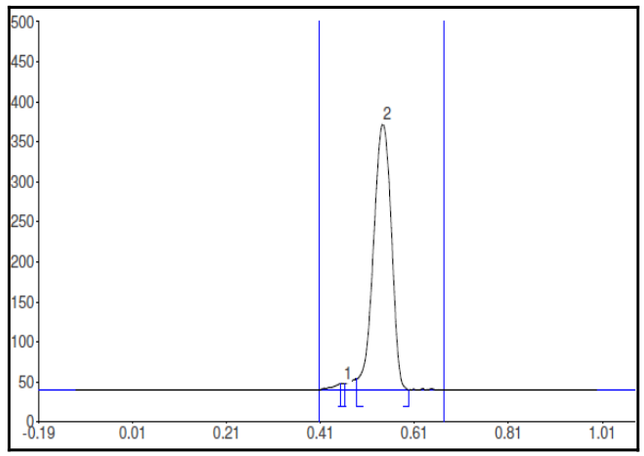


Figure 4. Track 3 - HPTLC peak of standard lupeol.

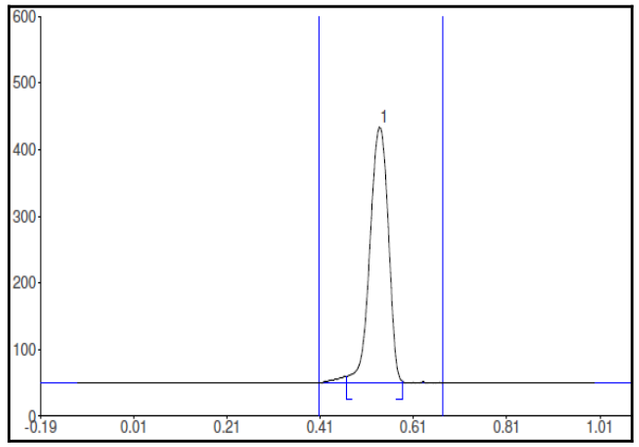


Figure 5. Track 4 - HPTLC peak of standard lupeol.

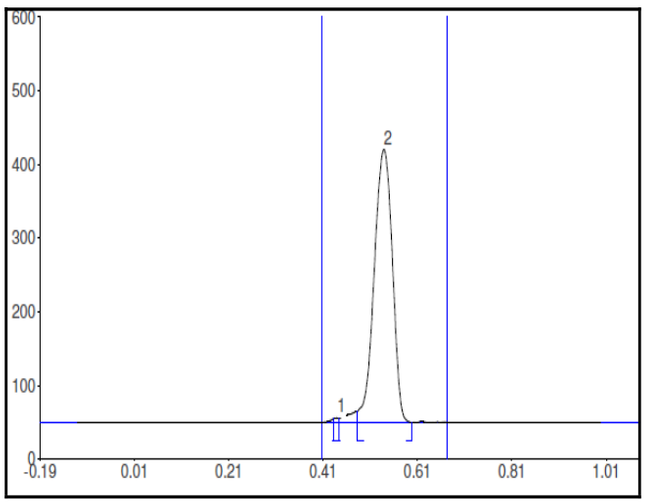


Figure 6. Track 9 HPTLC peak of standard lupeol.

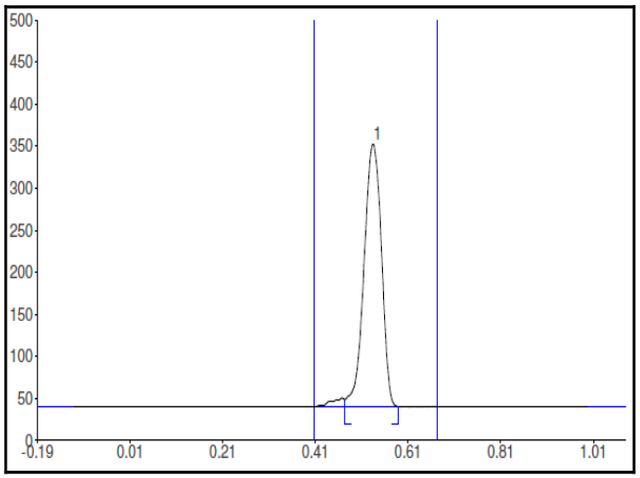


Figure 7. Track 10 - HPTLC peak of standard lupeol.

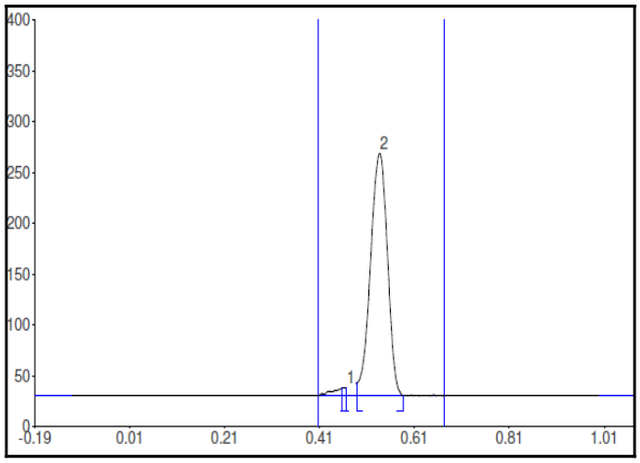


Figure 8. Track 11- HPTLC peak of standard lupeol.

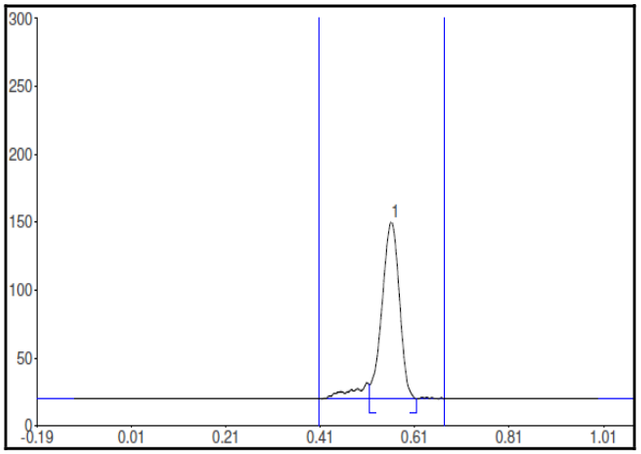


Figure 9. Track 12 - HPTLC peak of standard lupeol.

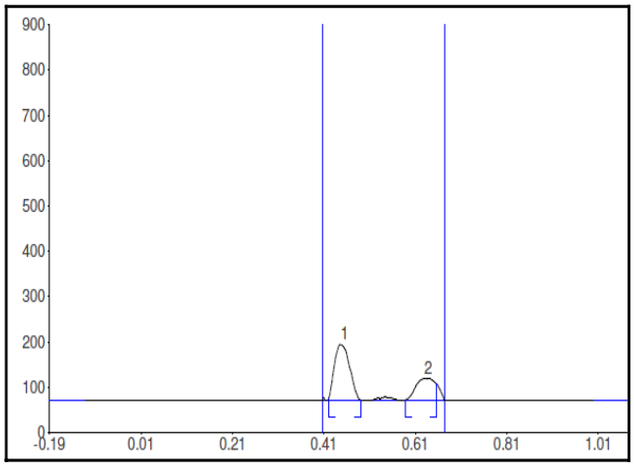


Figure 11: Track 6 - HPTLC peak of Benincasa hispida seeds extract.

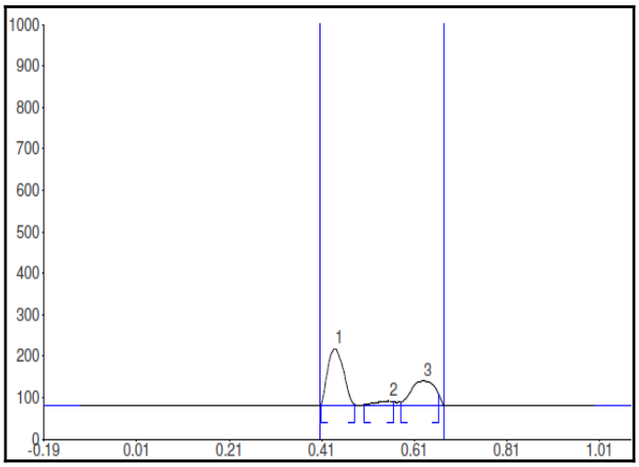


Figure 10. Track 5 - HPTLC peak of Benincasa hispida seeds extract.

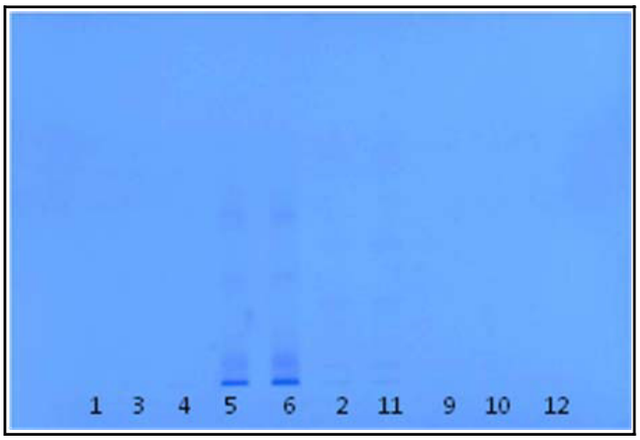


Figure 12. HPTLC Image before Derivatization.

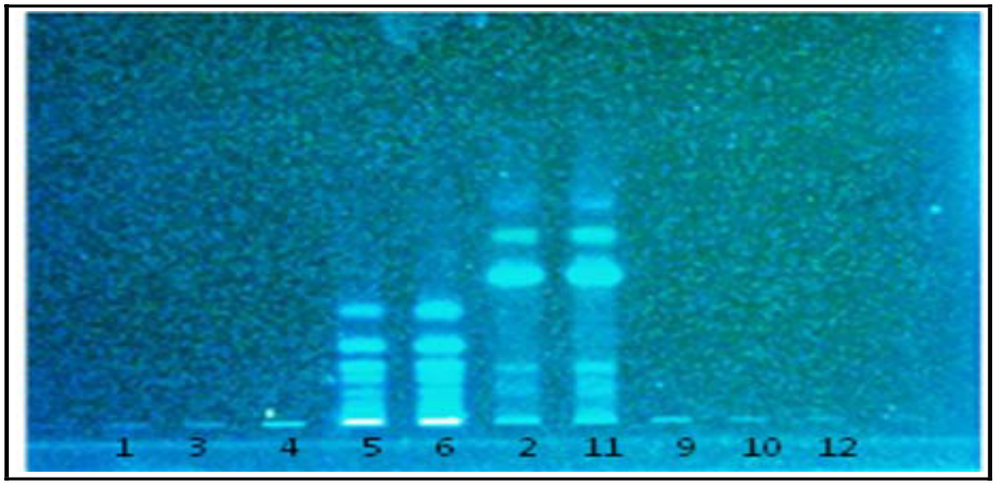


Figure 13. HPTLC Image after Derivatization.

(iii) HPLC reports

The petroleum ether BH seeds extract showed a characteristic retention peak (15.332 min) at 280 nm confirming the presence of lupeol at a flow rate of 1 ml/min using methanol solvent system (Table 3, Figures 14–15). The amount of lupeol present was **6.85% w/v**.

Table 3. HPLC analysis of Benincasa hispida and standard lupeol

Sample Name	Value
(Retention) Sample area of lupeol	(15.237) 9498.441
(Retention) Standard area of Lupeol	(15.532) 124739.67
Dilution of lupeol (Sample and Standard)	1:1
% of Lupeol	**6.85% w/v**

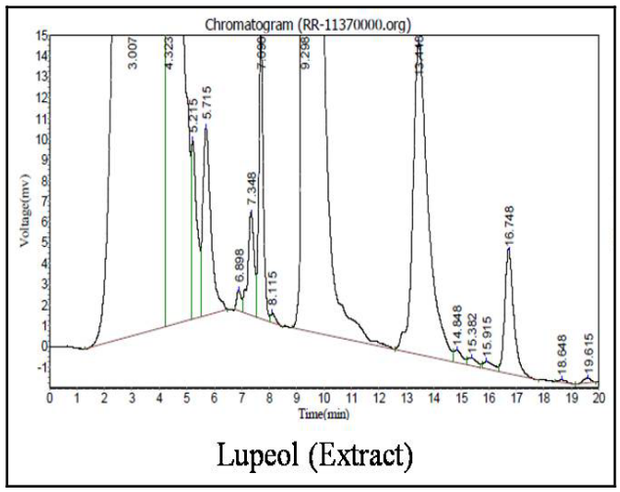


Figure 14: HPLC Peaks for Benincasa hispida extract.

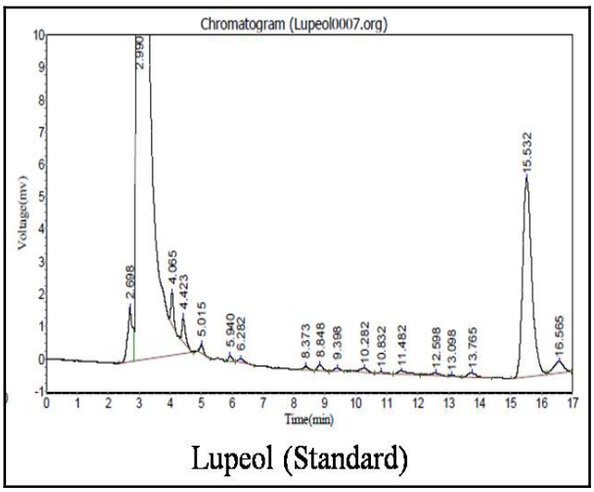


Figure 15: HPLC Peaks for standard lupeol.

DISCUSSION AND CONCLUSION

Lupeol has been found to play a vital role when obtained from different plant extracts reported in various studies. Our research studies draw the readers' vision towards the constituent in the petroleum ether *Benincasa hispida* seed extract confirmed by TLC, HPLC and HPTLC. The current research studies urge the young scientists to note the strong pharmacological prospects and correlate the same by undertaking studies on pharmacological models of different therapeutic categories.

ACKNOWLEDGEMENT

We would like to acknowledge the college management who provided us all the facilities to do this work as well as Radiant Research Services Pvt. Ltd. for their help in analysis.

REFERENCES

1. Ramakrishnappa K, Impact of cultivation and gathering of medicinal plants on Biodiversity: Case studies from India. Biodiversity and the Ecosystem Approach in Agriculture, Forestry and Fisheries FAO. 2002 February; Available from the Internet: http://www.fao.org/DOCREP/005/AA021E/AA02eoo.htm.
2. Anonymous. In: The Wealth of India: A Dictionary of Indian Raw materials and Industrial parts. Raw Materials Published by Council of Scientific & Industrial Research. Volume I: 82 & 173; 1948.
3. Doshi G, Shanbhag P and Une H, Rasayans and Non-rasayans: Future Immunodrug Targets. Pharmacog Rev. 2013; 14 7: 92–6.
4. Mandana B, Rahman AR, Farah ST, Mohd AN, Zaidul IS, Ali G, Optimization of Ultrasound-Assisted Extraction of Crude Oil from Winter Melon (*Benincasa hispida*) Seed Using Response Surface Methodology and Evaluation of its Antioxidant Activity, Total Phenolic Content and Fatty Acid Composition. Molecules 2012; 17: 11748–62.
5. Mazumder S, Morvan C, Thakur S, Ray B, Cell Wall Polysaccharides from Chalkumra (*Benincasa hispida*) Fruit. Part I. Isolation and Characterization of Pectins. J Agr Food Chem. 2004; 52: 35563562.
6. Latifah KD, Bioactive Proteins from *Benincasa hispida* (Thunb.) Cogn. Hayati J Biosci. 2009; 16 4: 161–4.
7. Pagare MP, Pati L, Kadam V,. *Benincasa hispida*: A Natural medicine. Res J Pharm Tech. 2011; 412: 1941–5.
8. Ghosh K, Baghel MS, A Pharmacognostical and Physiochemical study of *Benincasa hispida* with Ayurvedic Review. Int J Res Ayur Pharm. 2011; 26: 1664–1668.
9. Du Q, Qi Z and Ito Y, Isolation and Identification of Phenolic Compounds in the Fruit of *Benincasa hispida* by HSCCC. J Liq Chromatogr Rel Tech. 2005; 28: 137–44.
10. Gallo MBC , Sarachine MC, Biological Activities of lupeol. Int J Pharm Biomed Sci. 2009; 31: 46–62.
11. Oliveria EMS, Freitas SL, Martins FS, Couto RO, Pinto MV, Paula JR, Isolation and quantitative HPLC-PDA analysis of lupeol in phytopharmaceutical intermediate products from

Vernonanthura ferruginea (Less.). Quim Nova. 2012; 355: 1041–5.
12. Kokate CK, Practical Pharmacognosy, Vallabh Prakashan, New Delhi, India; 1989.
13. Khandelwal KR, Practical Pharmacognosy - Techniques and Experiments, 10th Edition; Nirali Prakashan, Pune; 2003.
14. Harborne JB, Phytochemical methods: A guide to Modern Techniques of Plant Analysis, Springer Publication House; 2005.
15. Nagore DH, Patil PS, Kuber VK, Comparison between High Performance Liquid Chromatography & High Performance Thin Layer Chromatography for determination of Diosgenin from the fenugreek seeds. Int J Green Pharm. 2012; 64: 315–20.

Evaluation of Antioxidant Activity of Five Medicinal Plants in Sri Lanka.

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Submission Date: 16-2-2014

Accepted Date: 24-3-2014

ABSTRACT

This study investigated on the antioxidant properties of five medicinal plants used in Sri Lanka, namely *Solanum nigrum*, *Amaranthus spinosus*, *Elephantopuss caber*, *Amorphophallus campanulatus* and *Canna indica*. The cold methanol plant extracts were screened for the antioxidant activity evaluating their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical in scavenging ability. The total ascorbic acid content of the extracts was also evaluated. The IC_{50} values of the extracts revealed that *Solanum nigrum* had the best DPPH scavenging activity with a value of 37.63 ± 0.11μg/ml and was better than that of the standard ascorbic acid. *Amorphophallus campanulatus* extract gave the highest ascorbic acid content of 143.03 ± 1.97 mg per 100 g of the extract. All five plants extract showed DPPH scavenging activity in the order of *Solanum nigrum* > *Elephantopus scaber* > *Amorphophallus campanulatus* > *Canna indica.* The plant extracts did not show a direct correlation between the ascorbic acid content to the DPPH scavenging activity. These experimental results reveals that these extracts can be utilized in future as therapeutic agent against free radical induced oxidative stress.

Keywords: Antioxidant activities, Ascorbic acid, DPPH, Medicinal Plants

INTRODUCTION

The history of use of plants in traditional medicine in Sri Lanka dates back into 4th century BC. Two of such medical systems as Ayurveda and Deshiya Chikitsa use mainly plant and herbal preparations for the treatment of diseases. There are over 2000 plant species used for medicinal purposes and many of these plants are endemic to Sri Lanka. The uses of such medicinal plants are documented and manuscripts can be found among Ayurveda doctors.[1] Many previous literature and practices reveal that plant material has been used as agents in curing and preventing many diseases as cancer, aging, skin diseases, cardiovascular disease, immune deficiency disease etc. due to their antioxidant properties.[2, 3]

Today, many medicinal plants in most countries are extensively investigated in search of novel drugs with antioxidants, antitumor, anti- mutagenic and antimicrobial activities. Many pharmaceutical industries are also involved in harnessing medicinally important natural products from plants. Around 60% of antitumor drugs and anti-infective drugs that are available in the market today are of the natural origin.[4] In the recent past the interest towards searching for antioxidant from natural products has increased due to potent and cost-effectiveness of the antioxidants from various plant sources.[5, 6] Furthermore use of antioxidant in prevention and treatment of cancer has provided many clinical benefits and therefore extensively used in inhibiting, or delaying carcinogenesis.[7, 8] It is also reported that many undesirable health effects are experienced from the use of existing synthetic antioxidants in both food and drugs. With the latest trend many food technologists add crude plant extracts into many food product with the intension of increasing the nutrient values, taste and as antioxidants.

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DOI: 10.5530/pj.2014.3.8

Antioxidants have the capability of inhibiting radical reactions that lead to undesirable conditions both in human and in food products. Free radicals and oxygen species such as, hydroxyl radicals, superoxides and other singlet oxygen are generated in the human body under physiological conditions. However all cells are equipped with enzymes as superoxide dismutase glutathione peroxidase and catalase as a natural defense system against the oxidative damage that could be caused by reactive radical and oxygen species under normal conditions. Excessive generation of such as reactive species are believed to be formed under disease conditions as cardiovascular disease, aging, and neurodegenerative diseases Alzheimer's disease, mutations and cancers.[9]

The plants exhibit their antioxidant activities due to naturally occurring compound as ascorbic acid, tocopherols, and polyphenols.[10] These antioxidants have been investigated and are used to protect the human body and food products from oxidative damage by inhibiting lipid peroxidation, scavenging free radicals and active oxygen species and chelating heavy metal ions.[11, 12] The addition of synthetic antioxidants, such as butylatedhydroxyanisole (BHA), butylatedhydroxytoluene (BHT), and tertiary butylhydroquinone has been widely used industrially. However, the uses of these synthetic antioxidants have been questioned due to their potential health risks and toxicity.[13]

The use of natural antioxidants for treating diseases and as food additives has better consumer acceptability and a trend over the use of the available synthetic products. Many research groups therefore have taken the responsibility of screening and quantification of the antioxidant activities of the medicinal plant

Therefore in this study we determined the *in vitro* antioxidant activities of five extracts of ayruvedic plant materials namely, *Solanum nigrum*, *Amaranthus spinosus*, *Elephantopus scaber*, *Amorphophallus campanulatus* and *Canna indica,* used in the Sri Lanka for many different medicinal purposes (Table 1). We determined their free radical scavenging activity against 1, 1-diphenyl-2-picryl hydrazyl (DPPH) and the total ascorbic acid content. It is important that this information on the antioxidant properties is available prior to incorporating them into food products and be used as drug for controlling diseases.

MATERIALS AND METHODS

Apparatus and materials

Spectrophotometer, 2, 2-diphenil-1-picrylhydrazyl (DPPH), Na_2CO_3, KIO_3, $Na_2S_2O_3{\cdot}5H_2O$, H_2SO_4, Soluble starch, Ascorbic acid, KI, $NaHCO_3$ (all chemicals used were in analytical grade or HPLC grade)

Plant Material: All plant materials were collected from the vicinity of University of Peradeniya, Peradeniya, Sri Lanka. Authenticity of the plant species was validated by the specific morphological and anatomical features from the authentic specimens available at the Sri Lanka Royal Botanical Gardens Peradeniya). The plant materials were air-dried at room temperature (26°C) for 2 weeks and thereafter grounded into a uniform powder for extraction.

Extraction: Methanol extracts were prepared by soaking 100 g of the dry powdered plant materials in 1L of methanol at room temperature for 48 h. The filtered extracts were concentrated under reduce pressure maintaining the temperature under 40°C.

DPPH Assay: The free radical scavenging ability of the extracts were determined using 2,2-diphenil-1-picrylhydrazyl (DPPH) radicals as describe in Braca, et. al., 2002.[14] Ethanolic DPPH solution (0.05 mM) was freshly prepared and kept in the dark at 4ºC until use. DPPH solution (300µl of 0.05mM) was added to 40µl of plant extract at different concentrations ranging from,12.5µg/ml to 100 µg/ml or into 40 µl of DI water (control). A volume of 2.7ml of Ethanol (96%) was added to the reaction mixture making the total volume to be 3.00 ml and was mixed vigorously. The mixture was left to stand for 30 min and the absorbance at 517 nm was measured. All experiments were performed in triplicate. The radical scavenging activities of the tested samples were expressed as percentage of inhibition was calculated according to the following equation.

Percent (%) Inhibition of DPPH Activity = $[(A_{control} - A_{sample})/A_{control}] \times 100$

The concentration of sample required for 50% inhibition was determined using linear regression analysis of the plot of extract concentration vs % inhibition and represented as $IC_{50}$of the extract. Lower IC_{50} value it indicates a greater antioxidant activity.

Determination of Total Ascorbic acid content: Ascorbic acid content was determined by the iodometric (redox) titration method. Fresh and dried plant material (10g) was added to 75ml of CO_2 free DI water and filtered into a 100 ml volumetric flask and adjusted the volume up to 100.00 ml using distilled water. A volume of 20 ml of 0.01 M KIO_3, 1 g of KI, 0.1 g of $NaHCO_3$ and 10 ml of 0.2 MH_2SO_4were added into the titration flask and thereafter 10ml of the above plant extract was added.

Table 1. Summary of medicinal uses of the plants investigated during this study

Botanical Name	Family	Common Name	Plant Part Used	Medicinal Uses
Solanum nigrum	solanaceae	Kalukanweriya" (Sinhala) Nightshade (English)	leaf	For gouty joints and rheumatism, piles, gonorrhea, dropsy and enlargements of the liver and spleen, sore eyes and various skin diseases
			fruit	treatment of malaria, black – water fever and dysentery (Rhodesia); for erysipelas (Mexico); diabetes (Philippines) fever, diarrhea, eye diseases and hydrophobia (Bengal); a substitute for raisins (Africa)
			plant	abdominal pain and inflammation of the bladder (Mauritius); headaches, ulcers, wounds and as a diuretic and emetic (Europe); antispasmodic, diaphoretic, emollient and sedative (Italians); as a vegetable (Africa)
Amaranthus spinosus	amranthaceae	Katuthampala (Sinhala) Prickly Amaranth –English	plant	Sudorific, febrifuge and eruptive fevers, piles (Ghana); a sudorific, febrifuge and galactagogue (Philippine); as a diuretic (Malaya & Mauritius)
			leaves	Often eaten as a pot herb good emollient &lactagogue properties for colic, locally for eczema.
			root	gonorrhea and mild diuretic and demulcent action
Elephantopus scaber	asteraceae	eth adi(Sinhala) Elephant's foot (English)	root	for urethral discharges, diarrhea, dysentery, dysuria and as a cardiac tonic (India); for cough (Malaya)
			plant	anthelmintic for roundworms and a decoction, decoction for increasing the discharge of urine (India, China); diuretic and febrifuge (Madagascar); as a tonic, diaphoretic and emmenogogue and given for dyspepsia (West Indies); decoction as a diuretic, febrifuge and emollient in the (Philippine Island)
			leaves	septic nails and wounds caused by bites of wild animals (Sri Lanka)
Amorphophallus campanulatus	araceae	kidaran (Sinhala) elephant foot yam (English)	Corm	tonic, stomachic, antibacterial, antifungal, appetizer and cytotoxic activity ,externally to relieve pain in acute rheumatism, eaten during periods of food scarcity, piles, acute dyspepsia, abdominal colic, elephantiasis, skin and blood diseases, fistula, glandular swelling in the neck, urinary disease and dropsy
			Roots	for boils and hemorrhoids, and ophthalmia, abdominal pains, tumors, spleen enlargement, asthma and rheumatism
			seed -	tooth – ache
			Tubers	hemorrhoids.
Canna indica	cannacea	Buthsarana (Sinhala) Indian–Bread Shot (Engish)	Root	diuretic, diaphoretic & demulcent. a food
			Seed	relieves ear ache, fever, dropsy, dyspepsia.

The mixture was titrated against 0.07 M $Na_2S_2O_3$ solution. Each extract was titrated in triplicates.[15]

RESULTS

DPPH Assay: The absorbance values at 517 nm, for the mixture of the extract with the DDPH showed a decrease with the increase in the concentrations of plant extract. These values were used to calculate the percentage of inhibition which showed an increase in the percentage inhibition with the increase in the concentration of plant extract (Figure 1). Using the plot, the IC_{50} value of each plant extract was evaluated. The IC_{50} of the plant extracts of *Canna indica, Amorphophallus campanulatus, Elephantopus scaber, Amaranthus spinosus and Solanum nigrum* were, 601.76 ± 2.31μg/ml, 136.39 ± 0.32μg/ml, 69.15 ± 0.08μg/ml, 56.51 ± 0.74μg/ml and 37.63 ± 0.11μg/ml respectively. The IC_{50} of the standard ascorbic acid was 40.71 ± 0.13μg/ml (Table 2; Figure 1). The plant extract

of *Solanum nigrum* showed the lowest IC_{50} value and which was lower than that of the standard ascorbic acid.

Ascorbic Acid Content

The ascorbic acid content in 100g of plant extracts as measured using the iodometic method revealed that *Amorphophallus campanulatus, Solanum nigrum, Amaranthus spinosus and Canna indica* contains 143.03 ± 1.97mg, 17.16 ± 1.78mg, 8.80 ± 1.81mg, and 0.43 ± 0.01mg respectively. *Amorphophallus campanulatus* showed the highest amount of ascorbic acid in its extract (Figure 2; Table 2).

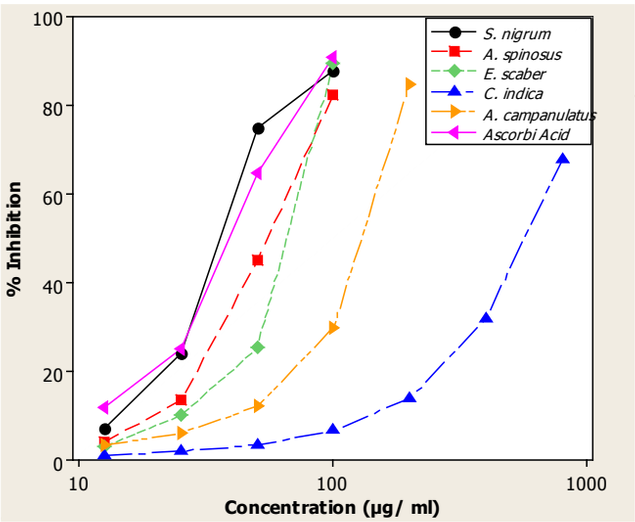

Figure 1. Antioxidant (DPPH scavenging) activity of investigated plant extracts as percentage of DPPH radicals inhibition and IC_{50} values (µg/ml).

DISCUSSION

In recent years, there has been a worldwide trend towards investigating on natural phytochemicals and quantifying their antioxidant properties. With many synthetic antioxidants exhibiting toxic and or mutagenic effects, the attention has shifted towards the use of naturally occurring antioxidants. Antioxidants have the capability of inhibiting or impairing radical reactions in many neurodegenerative diseases, cancers and AIDS.[16,17] Many plant extracts used in ayruvedha and herbal treatments has reported to have positive curing or controlling effects on the above diseases and many previous investigation have revealed that the many plant extracts does have antioxidant effects and could be therapeutically useful.[18]

During the present study five plants, namely, *Solanum nigrum, Amaranthus spinosus, Elephantopus scaber, Canna indica, and Amorphophallus campanulatus* were screened for

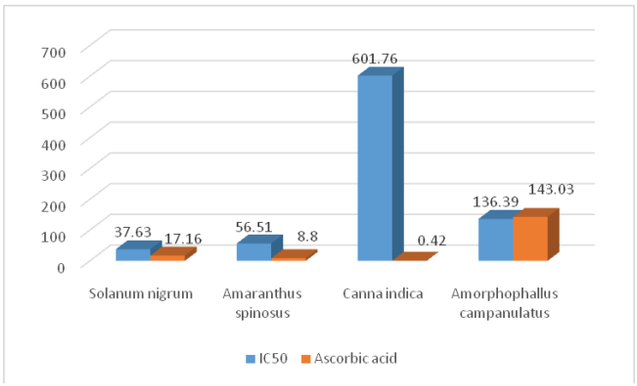

Figure 2. DPPH radical inhibition IC_{50} values (µg/ml) comparison with the ascorbic acid (mg/100 g extract) content in the investigated plant extracts.

Table 2. IC_{50} values (µg/ml) and ascorbic acid content (mg/100g of extract) in the methanol extracts of *Canna indica, Amorphophalluscampanulatus, Elephantopusscaber, Amaranthusspinosus and Solanumnigrum*

Plant	IC_{50} (µg/ml)	Ascorbic acid content (mg/100g of extract)
Canna indica	601.76 ± 2.31	0.43 ± 0.01
Amorphophallus campanulatus	136.39 ± 0.31	143.03 ± 1.97
Elephantopus scaber	69.15 ± 0.08	ND
Amaranthusspinosus	56.51 ± 0.74	8.80 ± 1.81
Solanum nigrum	37.63 ± 0.11	17.16 ± 1.78
Ascorbic acid	40.71 ± 0.13	NA

ND- Not determined NA- Not applicable

their antioxidant properties by evaluating their free radical scavenging activity and also quantifying the ascorbic acid content in the extract.

The model DPPH free radical scavenging assay is an easy method to evaluate antioxidant activity in a relative short time compared to the other methods.[19] DPPH is a relatively stable radical. The assay is based on the measurement of the scavenging ability of the antioxidants towards the stable radical DPPH which reacts with suitable reducing agent. The electrons become paired off and solution loos its color stoichiometrically depending on the number of electrons taken up. The color change can be quantified by its decrease of absorbance at wavelength 517nm. The antioxidants exert their DPPH free radical scavenging due to their hydrogen donating ability.[18]

The decreasing order of the IC_{50} Values of the plant extracts were as *Canna indica* > *Amorphophallus campanulatus* > *Elephantopus scaber* > *Amaranthus spinosus* > *Solanum nigrum* (Table 2; Figure 1). The results clearly indicated that *Solanum nigrum* is the plant extract with the best DPPH scavenging activity with an IC_{50}value of 37.63 ± 0.11μg/ml and even better than that of ascorbic acid (40.71± 0.13μg/ml). The uses of the plant has been different form country to country and it is reported a wide range of uses as to treat sore eyes, various skin diseases, malaria (Rhodesia), diabetes (Philippines), inflammation of the bladder (Mauritius), headaches, ulcers, wounds (Europe) and many more,[20] (Table 1). The lowest DPPH activity was recorded form *Canna indica* during this experiment yet the plant extract is widely used for its antioxidant properties in traditional medicinal uses. Previous studies performed on *Canna edulis* (cold methanol extract) showed an IC_{50}value of 570μg/ml and it is believed that the activity is due to the presence of polyphenols and flavonoids. This plant is also widely used for its antioxidant properties.[21]

Ascorbic acid which is commonly known as Vitamin C is required for the prevention of scurvy and maintenance of healthy skin etc. Its deficiency would also lead to hemorrhage of mucus membrane of the mouth and gastrointestinal tract, anemia, pains in joint.[22] It also plays a major role in activity of the enzyme prolyl hydroxylase which synthesizes 4-hydroxyproline, an amino acid that is required in collagen formation. It is believed that ascorbic acid helps in the enhancement of the immune system. It also scavenges free radicals by acting as a chain breaking antioxidant that impairs the formation of free radical, quenches O_2^-, and acts as a reducing agent and thereby reducing the risk of arteriosclerosis, cardiovascular diseases and some forms of cancer.[16,17, 23]

All plant exractsof *Amorphophallus campanulatus, Solanum nigrum, Amaranthus spinosus and Canna indica* showed significant amount of ascorbic acid in their extracts. However *Amorphophallus campanulatus* showed the highest amount of ascorbic acid in its extract (Figure 2; Table 2). Previous studies performed in India on the same plant recorded to contain 14.5 μg of ascorbic acid per milligram of extract indicating the values obtained in our experiment to be very much similar.[24] The plant extracts of *Amorphophallus campanulatus* has reported to be have many medicinal uses as antibacterial, antifungal, and cytotoxic activity. It is also used to relieve pain in acute rheumatism, skin & blood diseases, abdominal pains, tumors, spleen enlargement, asthma and rheumatism.[20] It is also widely studied for its activity against induce oxidative stress[25] and hepatoprotective activity[26] in the recent past and has exhibited promising results for the same.

Correlating the ascorbic acid content with the antioxidant activity as determined by DPPH assay did not show a direct correlation between each other (Figure 2) indicating that ascorbic acid is not, the only substance involved in radical scavenging activity. However extract of *Solanum nigrum* with its substantially low IC_{50} value for the inhibition of DPPH radicals and *Amorphophallus campanulatus* for the presence of ascorbic acid has shown promising results as good natural antioxidant. These values greatly supports the investigations results reported on *Amorphophallus campanulatus* in the resent past for its activity against induce oxidative stress[25] and hepatoprotective activity.[26, 27]

CONCLUSION

With the potential of using natural antioxidants as medicines and as food additive the antioxidant research has attracted a prominent place at present. With the reported undesirable effects of synthetic antioxidants and the high cost for such antioxidants, have also encouraged most of the people to use natural antioxidants.

Through this investigation we have shown that all five plants used in this investigation exhibits antioxidant activities with four of them namely to *Solanum nigrum, Amaranthus spinosus, Elephantopus scaber, and Amorphophallus campanulatus* be having remarkable antioxidant activity with a good potential to be used in therapeutics. *Solanum nigrum* revealed to be the most potent plant extract with the best radical scavenging activity and a substantial amount of ascorbic acid. These results also elaborate on the ascorbic acid content that could be used effectively

in the traditional or combinational medical practices. Since the results also show that it is not only the ascorbic acid is responsible in the antioxidant activities of these extracts must be further investigated in quantifying the other responsible antioxidant. The results presented in this report will also provide a suitable guide in choosing natural plant by the medical practitioners as natural oxidants treating and controlling diseases.

REFERENCES

1. Weragoda PB.1980. The traditional system on medicine in Sri Lanka. Ethanophamacol.21: 71–83.
2. Middeleton E, Kandaswamy C, Theoharides TS. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmacol Rev. 2000; 52 :673–751.
3. Ishikawa Y, Morimoto K, Hamasaki T, Falvoglaucin, a metabolite of *Eurotium chevalieri*, its antioxidation and synergism with tocopherol. J. Am. Oil Chem. Soc. 1984; 61:1864–1868.
4. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J. Nat. Prod. 1997; 60: 52–60.
5. Liu F, Ng TB. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sciences, 2000; 66: 725–735.
6. Park EJ, Pezzuto JM. Botanicals in cancer chemoprevention. Cancer and Metastasis Rev. 2002; 21: 231–255.
7. Shureiqi I, Reddy P, Brenner DE. Chemoprevention: General perspective. Critical Rev.in Oncology/Hematology. 2000; 33: 157–167.
8. Tsao AS, Kim ES, Hong WK. Chemoprevention of Cancer, CA-A Cancer Journal for Clinicians, 2004; 54: 150–180.
9. Koppula SB. Ammani K. Antioxidant findings of Araku environment medicinal plants using different assays.Drug invention today. 2011; 38**:** 203–205.
10. Duh, PD, Tu YY, Yen GC. Antioxidant activity of water extract of Harng Jyur (*Chyrsanthemum morifolium* Ramat). LWT-Food Sci. Technol. 1999; 32: 269–277.
11. Akula V, Odhar B. In vitro 5-lipoxygenase inhibition of polyphenolic antioxidants from undomesticated plants of South Africa. Journal of Medicinal plants research, 1999; 22: 207–209.
12. Sudarajan N, Ahamad H Kumar V. Cytisusscoparius Link- A natural antioxidant 2006; 6: 1–7.
13. Kahl R, Kappus H, Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin E. Z LebensmUntersForsch. 1993; 1964: 329–38.
14. Braca A,Sortino C, Politi M, Morelli I, Mendez J. Title missing J, of Ethnopharmaco. 2002; 79: 379–381.
15. Dioha IJ, Olugbemi O, Onuegbu TU, Shahru Z. Determination of ascorbic acid content of some tropical fruits by iodometric titration. Int. J. Biol. Chem. Sci. 2011; 55: 2180–2184.
16. Beyer RE. The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. J. Bioenerg. Biomembr. 1994; 26: 349–358.
17. Aqil F, Ahmad I, Mehmood Z. Antioxidant and Free Radical Scavenging Properties of Twelve Traditionally Used Indian Medicinal Plants. Turk. J. Biol. 2006; 30: 177–183
18. Kannat SR, Chander R, Sharma A. Antioxidant potential of mint (*Mentha spicata* L.) in radiation-processed lamb meat. Food. Chem. 2007; 600: 451–458
19. Koleva II, Van Beek TA, Linssen JP HA, de Groot, Evstatieva, LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis 2002; 13: 8–17.
20. Jayaweera DMA. Medicinal Plants Used in Ceylon, Part 1-5, National Science Foundation, Sri Lanka.
21. Mishra T, Goyal KA, Middha SK, Sen A. Anti-oxidative properties of *Canna edulis* Ker – Gawl., Indian Journal of Natural Products and Resources. 2011; 23 : 315–321.
22. Iqbal K, Khan A, Khattak MMA. Biological Significance of Ascorbic Acid (Vitamin C) in Human Health – A Review. Pakistan Journal of Nutrition. 2004; 31: 5–13.
23. Brewer MS, Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications Comprehensive Reviews in Food Science and Food Safety 2011; 104: 221–247.
24. Raghu KL, Ramesh CK, Srinivasa TR, Jamuna KS. Total Antioxidant Capacity in Aqueous Extracts of Some Common Vegetables. Asian J. Exp. Biol. Sci. 2011; 21: 58–62.
25. Ansil PN, Prabha SP, Nitha A, Wills PJ, Jazaira V, Latha MS, Curative effect of *Amorphophallus campanulatus* (Roxb.) Blume tuber methanolic extract against thioacetamide induced oxidative stress in experimental rats. Asian Pacific Journal of Tropical Biomedicine. 2011; S83–S89.
26. Jain S, Dixit VK, Malviya N, Ambawatia V. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Amorphophallus campanulatus* Roxb. tubers. Acta Pol Pharm. 2009; (64): 423–8.
27. Sanjay J, Vinod K,Dixit NM, Ambawatia V. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Amorphophallus campanulatus* roxb. Tubers. Acta Poloniae Pharmaceutica and Drug Research, 2009; 66 4: 423–428.

Anti-inflammatory and hepatoprotective activities of methanolic extract of *Anthemis scrobicularis* herbs

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ABSTRACT

The anti-inflammatory and hepatoprotective activitiesof the methanolic extract of *Anthemis scrobicularis*(ANS) herbs were evaluated in rats against carrageenan induced inflammation and carbon tetrachloride (CCl_4)induced hepatic injury. To evaluate the anti-inflammatory effects of ANS, twenty male rats were divided into four equal groups. Injection of 100 µl carrageenan in normal saline into the subplantar region of the hind paw of rats clearly induced paw edema. The volume of paw edema was attenuated following oral administration of ANS. For hepatoprotective effects, twenty five rats were equally divided into five groups.The hepatotoxicity, induced by a single dose of CCl_4, produced significant ($p < 0.001$) increase of the levels of serumtransaminase, phosphatase, bilirubin and a decrease in proteins were also noticed. The oxidative stress marker such as malondialdehyde (MDA)was increased and non-protein sulfhydryl (NP-SH) was decreased in the hepatotoxic tissues. Pre-medication of CCl_4-intoxicated rats with ANS at the doses 250 and 500 mg/kg reversed the abnormal liver diagnostic stricture. The results showed that ANS is toxicologically safe when orally administered and possess highly significant anti-inflammatory and hepatoprotective activities and the potentials usefulness of *Anthemis scrobicularis* in hepatic and inflammatory disease.

Key words: *Anthemis scrobicularis*, Anti-inflammatory, Hepatoprotective, Carbon tetrachloride, Histopathology.

INTRODUCTION

Anthemis is the second largest genus of the tribe Anthemideae, comprises of nearly 210 species.[12] It is represented in Saudi Arabia by 19 species.[3] Anthemis scrobicularis Yavin, Fam. Asteraceae is an annual herb, growing in sand dunes and sandy areas, Arabian peniusula Jordan, and Palestine.[3] There is wide interest in research of the plants of Anthemis, especially their active components, because many of the plants are reported to have antifungal[4] antioxidant,[5] antitumour, antiplasmodial, anthelminthic, schistosomicidal, cytotoxic, phytotoxic, analgesic activities[6] and for treatment of cystitis and dental afflictions.[7] Extracts, tinctures, salves, tisanes, infusion, decoction and other traditional formulations of same or related species are widely used for treatment of inflammation, dysmenorrhoea, hepatotoxicity, hemorrhoid, abdominal pain and different types of skin inflammation in the European folk medicine.[8–11] The occurrence of sesquiterpene lactones, flavonoids, sterols, fatty acids, polyacetylenes and essential oils in various Anthemis species has been reported in previous works.[12–16] These phytochemicals have been previously reported for anti-inflammatory, antioxidant and hepatoprotective activities.[17–22] Nonsteroidal anti-inflammatory drugs are among the most common drugs associated with drug-induced liver injury, with an estimated incidence of between 3 and 23 per 100,000 patient and the drugs like Nimesulide, sulindac, and diclofenac seem to be associated with the highest risk factor.[23] As per our knowledge, there is no previous report on the activity of this plant. In the present study, we report the safety, anti-inflammatory and anti-hepatotoxicity effectiveness of the methanolic extracts of Anthemis scrobicularis for the first time.

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DOI: 10.5530/pj.2014.3.9

MATERIALS AND METHODS

Materials

All the chemical and reagents procured were analytical grade. Carrageenan was purchased from BDH Chemicals Ltd., UK, while other chemicals were purchased from Sigma Aldrich.

Collection and Authentication of plant

The aerial parts of *Anthemis scrobicularis* Yavin, Fam. *Asteraceae* collected from the sandy areas near AlKharj governorate, Saudi Arabia in April 2013. The plant was kindly authenticated by Dr. Yousef Yaquob, College of Pharmacy, Department of Pharmacognosy, King Saud University, Al-Riyadh, Kingdom of Saudi Arabia. The plant material was air dried and reduced to fine powder.

Extraction

Powdered *Anthemis scrobicularis* (2 Kg) was extracted by percolation in methanol (3 × 4L) at room temperature for 3 days. The combined methanolic extract was concentrated in rotary evaporator at 45°C to 500 ml and then diluted with distilled water (500 ml). Lead acetate solution was then added drop wise until no more precipitate is formed then filtered. The filtrate was extracted with chloroform (4 × 200 ml) and concentrated in rotary evaporator at 45°C to afford yellowish brown residue (20 g).

Toxicological and Pharmacological evaluation:

Swiss albino mice (25–30 g b. wt) and Wistar albino rats (200-250g) of both sexes were used for anti-inflammatory and hepatoprotective studies. The animals were obtained from Lab Animal Care Unit, Pharmacy College, King Saud University, Riyadh, KSA. The animals were housed in the animal house of the Department of Pharmacology, College of Pharmacy, Salman Bin Abdulaziz University, Al-Kharj, KSA for acclimatization. The animals were kept in groups of five per cage under standard environmental conditions of temperature and light/dark (12/12 h) cycles, and provides commercial rat or mice feed and tap water given *ad libitum*. The animals were allowed to acclimatize to the laboratory condition for one week before commencement of the experiment. The experiments and procedures used were approved by the Ethical Committee of the College of Pharmacy, King Saud University, Riyadh.

Determination of acute toxicity and median lethal dose (LD50) of the extracts

LD_{50} of *A. scrobicularis* were determined according to the reported method.[24] Mice were divided into groups of 5 animals and the tested extracts were administered orally in doses of 0.1 to 5g/kg body weight. Signs of acute toxicity and number of death per dose within 24 h were recorded and the LD_{50} was calculated.

Carrageenan-induced rat hind paw edema

Wistar rats were fasted for 16 h and were divided into 4 groups, each containing 5 individuals.[25] The control group was given 5 ml/kg of normal saline. The test groups of rats were treated orally by 250 and 500mg/kg ANS separately.The reference group was given 100mg/kg of an aqueous solution of phenylbutazone. One hour later, paw edema was produced by injecting 100 µl of 1% solution of carrageenan in saline into the left hind paw. Paw volume was measured before and after carrageenan injection up to 3 h, using a water displacement plethysmometer (plethysmometer (Ugo Basile 7150)).

Hepatoprotective study

The Hepatoprotective activity was evaluated in Wister albino rats using CCl_4 induced liver injury.[26] The rats were divided into 5 groups (5 animals each) and were treated in accordance, Group-one was served normal saline (control); Group-two was served 1.25ml/kg of CCl_4 (hepatotoxic), Group-three and four were served 250 and 500 mg/kg b.w. respectively (ANS extracts), Group-five was served silymarin (positive control). The animals were killed under light ether anesthesia 24 h after the last treatment. Blood was collected by cardiac puncture in plain tubes and their livers and kidneys were removed immediately after necropsy. Serum was separated by centrifugation at 3000 rpm at 4°C for 10 min. 10% (w/v) liver homogenate was prepared in 0.25M sucrose solution and centrifuged at 7000 rpm for 10 min at 4°C.

Assessment of liver function

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by Reitman and Frankel methods,[27] serum alkaline phosphatase (ALP] levels were estimated by King and Armstrong method,[28] gamma glutamyl transpeptidase (GGT) activity was determined by Szas method[29] and the bilirubin level in serum was determined by modified DMSO method of Walters and Gerarde.[30] The protein concentration was determined according to the Lowry et al., method[31] using bovine serum albumin (BSA) as a standard.

Assessment of oxidative stress

Malondialdehyde (MDA) was determined by Ohkawa et al., methods[32] and Non-Protein sulfhydryls (NP-SH) was determined by according to the Sedlak and Lindsay method[33] in liver tissue.

Histopathological studies of liver and kidneys

Liver and kidneys were perfused with cold saline at 4°C and excised immediately. A small fragment of liver and kidney tissues was placed in 10% formalin (diluted to 10% with normal saline) for 1 hr. For histological studies, a portion of the liver or the kidney was fixed in ascending grades of isopropyl alcohol by immersing in 80% isopropanol overnight and 100% isopropyl alcohol for 1 hour and finally paraffin wax (four times 1h). Tissues were transferred in to paraffin waxed filled moulds. The rotary microtome (Leitz 1512) was used for making the section (3 µm). The sections were placed on clean slides and place onto warming table at 37-40^{0}C. The slides were stained for 15min with Mayer's hematoxylin solution; washed for 15 minutes in lukewarm running tap water then distilled water for 2 minutes, then finally with 80% ethyl alcohol.The slides were then counter stained for 2 minutes with eosin-phloxine solution. Histological observations were made under light microscope.

Statistical Analysis: Data recorded was analyzed as mean ± SEM (standard error of mean) in each group. Differences between groups were determined by unpaired Student's *t*-test.

RESULT

Toxicity study

The results indicated that different doses of ANS (up to 5000 mg kg^{-}) did not produce any symptoms of acute toxicity.

Anti-inflammatory activity:

Oral administration of the crude extract of ANS (250-500 mg/kg) caused significant (P<0.001) inhibition of edema induced by the injection of carrageenan.

In (tabl-1), the carrageenan-induced rat paw edema at 2h was 2.17±0.02 mL. The mean reduction in rat paw edema carrageenan with phenylbutazone (PBZ) was 1.28±0.04 mL. The mean reduction in rat paw edema of 250 and 500mg/kg ofANS extracts was 1.70±0.05 and 1.34±0.03 mL respectively.

Assessment of liver function

The examination of ALT, AST and GGT were given in **(**Table 2**)**. CCl_4 (1.25ml/kg) significantly (p<0.001) elevated the serum activities of ALT, AST and GGT when compared to the normal saline animals. Administration

Table 1. The effect of ANS on carrageenan induced hind paw edema in rats

Treatment	Dose mg/kg	Increased in rat paw edema (ml±SEM)		Net Reduction	Percentage inhibition
		0h	2h		
Carrageenan (1%)	0.1 ml/kg	1.05 ± 0.03	2.17 ± 0.02	1.12 ± 0.02	---
A. scrobicularis+ Carrageenan	250	1.08 ± 0.03	1.70 ± 0.03	0.61 ± 0.02***	45.02
A. scrobicularis + Carrageenan	500	0.98 ± 0.04	1.34 ± 0.03	0.36 ± 0.03***	63.06
PBZ+ Carrageenan	100	1.08 ± 0.04	1.28 ± 0.04	0.19 ± 0.001***	82.61

Values are mean ± SEM. n=5, ***P< 0.001. Ananti-inflammatory extract of *A. scrobicularis* + CCl_4 (250 and 500mg/kg, bw) showed a statistically significant result.

Table 2. Effect of ANS on serum activity of ALT, AST and ALP in CCl_4-intoxicated rats

Treatment	AST (IU/l)	ALT (IU/l)	GGT (IU/l)
Normal saline (2ml/kg, p.o.)	75.40 ± 13.26	32.86 ± 2.39	4.70 ± 0.28
CCl_4 (1.25ml/kg,i.p.)	211.33 ± 10.08***	194.16 ± 7.53***	11.25 ± 0.37***
A. scrobicularis +CCl4 (250mg/kg, p.o.)	153.50 ± 5.51***	134.66 ± 2.45***	7.76 ± 0. 19***
A. scrobicularis +CCl4 (500mg/kg,p.o.)	134.00 ± 4.19***	123.66 ± 5.04***	6.65 ± 0.18***
Silymarin(10 mg/kg, i.p.)	124.16 ± 7.13***	112.93 ± 6.13***	5.83 ± 0.25***

Values are mean ± SEM. n=5, ***P< 0.001. A Hepatoprotective extract of *A. scrobicularis* + CCl_4 (250 and 500mg/kg, bw) showed a statistically significant result.

of ANS at doses of 250 and 500mg/kg prior to CCl_4 significantly protected against the elevation of transaminases levels. The serum activities of AST, ALT and GGT in rats treated with ANS extract at a dose of 250 mg/kg + CCl_4 were 153.50±5.51, 134.66±2.45, and 7.75±0.18 IU/l, respectively and with 500 mg/kg plus CCl_4 were 134.00±4.19, 123.66±5.04 and 6.65±0.18 IU/l, respectively. These values were highly significant when compared with the intoxicated control rats (211.33±10.08, 194.16±7.53 & 11.25±0.37 IU/l respectively). Similarly, the elevated levels of serum ALP, bilirubin and total protein oftissue were significantly decreased in ANS extract (Table 3).

Malondialdehyde and NP-SH assays

The effect of ANS on the CCl_4-induced lipid peroxidation was examined through observation of the levels of MDA in liver tissues. Hepatic MDA and NP-SH level were significantly ($p<0.001$) changed in the CCl_4-intoxicated control group (5.10±0.3 nmol/g tissue and 1.10±0.09nmol/g of tissue) than the normal animals (1.077±0.05 nmol/g and 5.86±0.37nmol/g tissue). Treatment with ANS (250 & 500 mg/kg) andCCl_4 significantly ($P<0.001$) prevented the changed of MDA and NPSH. Silymarin treatment also prevented the CCl_4 Changed MDA (2.04±0.12 nmol/g tissue) and NP-SH(1.81±0.10nmol/g tissue) (Table-4).

Liver and kidney histopathological studies

Histopathological studies of liver of animal in the normal saline control group showed normal hepatic architecture (fig 1a), where the hepatocytes showed cytoplasmic vacuolization of hepatocytes and partial infiltration with inflammatory cells (fig 1b). Treatment with Silymarin and ANS (250 & 500 mg/kg) exhibited reversal of these changes (Figure 1c, d, e). However, protective effects were more pronounced at higher dose of ANS (500 mg/kg bw) and revealed no histological changes.Histopathological studies of kidney in saline control group showed normal histological structure of renal parenchyma (fig 2a), where the hepatocytes showed cytoplasmic vacuolization of epithelial lining renal tubules (fig 2b). Treatment withsilymarin and ANS (250 & 500 mg/kg) exhibited reversal of these changes (Figure 2c, d, e).

DISCUSSION

The extract of ANS did not produce any symptoms of acute toxicity in mice, so the extracts are safe for animal's use. Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and the probable mechanism of action is bi-phasic; the release of histamine, serotonin, 5-HT and kinins in the first phase; while swelling is related to the release of

Table: 3. Effect of ANS on serum activity of GGT and bilirubin and tissue activity of total protein in CCl_4-intoxicated rats

Treatment	ALP (IU/l)	Bilirubin (mg/dl)	Total protein (g/l)
Normal saline (2ml/kg, p.o.)	294.33 ±6.17	0.54 ±0.02	125.51 ±3.54
CCl_4 (1.25ml/kg,i.p.)	514.16 ±11.75***	2.34 ±0.16***	56.55 ±3.47***
A. scrobicularis +CCl4 (250mg/kg, p.o.)	405.33 ± 9.50***	1.50 ±0.04***	94.71 ± 3.31***
A. scrobicularis +CCl4 (500mg/kg,p.o.)	340.66 ±4.19***	1.17 ±0.05***	111.26 ±3.94***
Silymarin(10 mg/kg, i.p.)	9.90.16 ±9.77***	0.90 ±0.06***	92.41 ±3.54***

Values are mean ± SEM. n=5, ***P< 0.001. A Hepatoprotective extract of *A. scrobicularis* + CCl_4 (250 and 500mg/kg, bw) showed a statistically significant result.

Table 4. Effect of ANS on MDA and NP-SH in liver tissue of rats with CCl4 induced hepatotoxicity

Treatment	MDA (nmol/g)	NP-SH (nmol/g)
Normal saline (2ml/kg, p.o.)	1.077±0.05	5.86±0.37
CCl_4 (1.25ml/kg,i.p.)	5.10±0.3***	1.10±0.09***
A. scrobicularis +CCl4 (250mg/kg, p.o.)	3.28±0.14***	2.01±0.18***
A. scrobicularis +CCl4 (500mg/kg,p.o.)	1.99±0.11***	2.43±0.16***
Silymarin(10 mg/kg, i.p.)	2.04±0.12***	1.81±0.10***

Values are mean ± SEM. n=5, ***P< 0.001. An Antioxidant extract of *A. scrobicularis* + CCl_4 (250 and 500mg/kg, bw) showed a statistically significant result.

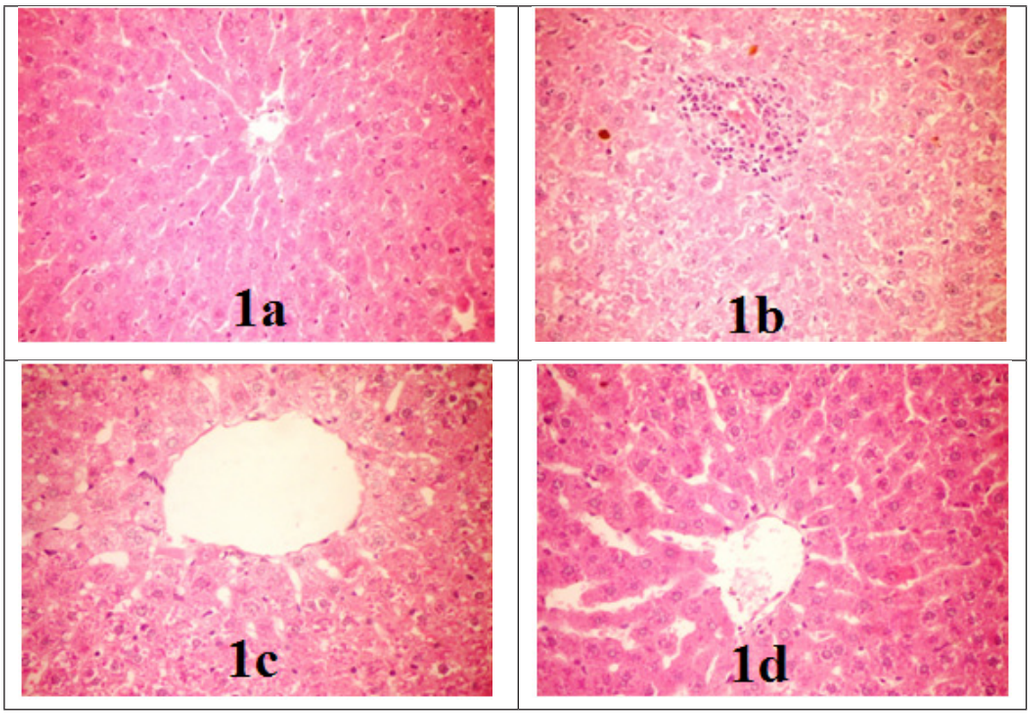

Figure 1. Histopathological section of liver tissue of rats (H & E×400). **(1a)** Section of control rat showing the normal histology, **(1b)** Section of CCl_4 induced hepatotoxic rat showing cytoplasmic vacuolization of hepatocytes and partial infiltration with inflammatory cells. **(1c)** ANS + CCl4 (250 mg/kg bw) showing slight granularity of the cytoplasm of hepatocytes and **(1d)** ANS + CCl_4 (500 mg/kg bw) showing no histopathological changes.

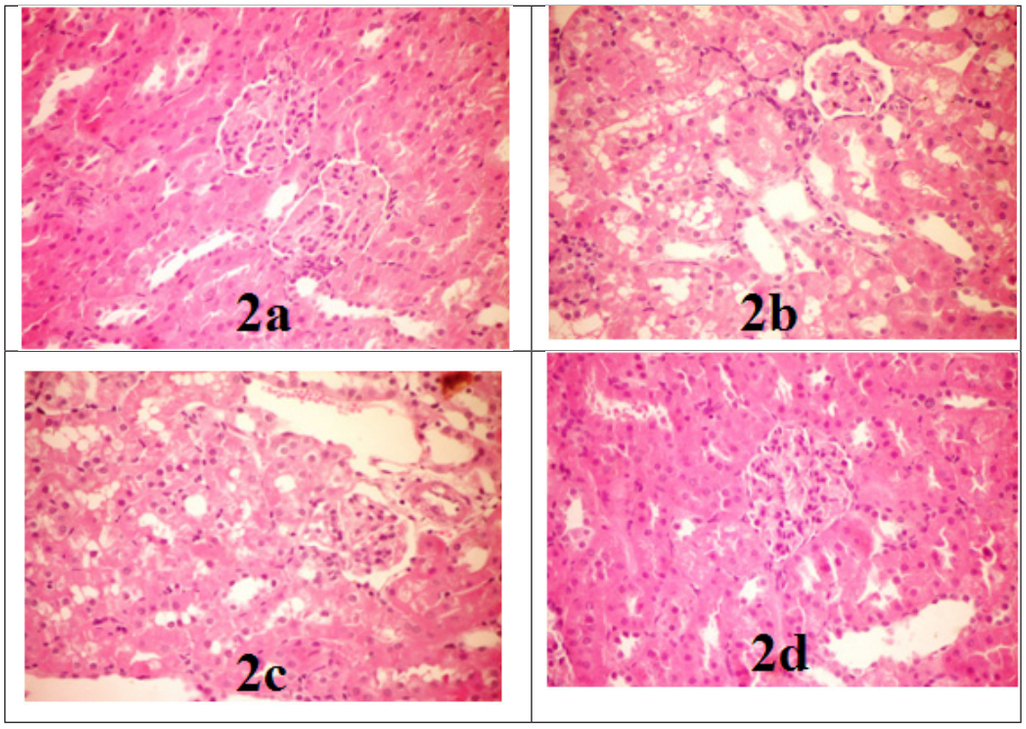

Figure 2. Histopathological section of renal tissues(H & E×400).**(2a)** Section of control rat showing the normal histology, **(2b)** Section of CCl_4 induced renal toxicity showing vacuolization of cytoplasm of epithelial lining renal tubules. **(2c)** ANS + CCl4 (250 mg/kg bw) showing vacuolar degeneration of epithelial lining of some renal tubules and **(2d)** ANS + CCl_4 (500 mg/kg bw) showing no histopathological changes.

prostaglandin, bradykinins and lysozymes-like substances in 2-3 h in the second phase.[34] The anti-inflammatory effect of the crude extract of the plant at the third hour after carrageenan injection strongly suggests its NSAID like activity. Similarly the standard drugs, diclofenac and indomethacin, produced a significant anti-edematous effect showed marked inhibition of carrageen induced edema in rats.[35] Some of related species has already been reported for anti-inflammatory effects.[10,20,21] Carbon tetrachloride-induced hepatic injury is commonly used as an experimental method for the evaluation of hepatoprotective drugs or medicinal plant extracts.[36] Generally, the extent of hepatic damage is assessed by histopathological evaluation and the level of cytoplasmic enzymes released into the circulation. Marked elevation of serum enzymes and total protein, MDA and NP-SH in liver tissue indicates damage to the hepatic tissue. The disturbance in the transport function of the hepatocytes, as a result of hepatic injury, causes the leakage of enzymes from cells due to altered permeability of the membrane that result in raised levels of enzymes.[37] The normalization of the level of the corresponding enzymes is a definite indication of the hepatoprotective action of the compound under evaluation. ALT and AST are the most sensitive markers of hepatocellular injury and their elevation in serum is indicative of cellular leakage and loss of the functional integrity of cell membranes in liver.[38] ALP is a membrane bound enzyme involved in active transport across the capillary wall. The increased level of ALP is also a reliable marker of liver damage.[39] GGT is important in transport of amino acids required for the synthesis of GSH in cells. Bilirubin is an important degradation product of hemoglobin and is normally excreted into the bile. Increase in total serum bilirubin concentration after CCl_4 administration might be attributed to the failure of normal uptake, conjugation and excretion by the damaged hepatic parenchyma. Decline in enzymes levels after *A. scrobicularis* administration indicated improvement in cellular integrity and status of hepatic cells. Non-protein sulfhydryls are known to be involved in several defense processes against oxidative damage; protect cells against free radicals peroxides and various poisonous substances.[40] The increased TBARS after CCl_4 administration suggests enhanced LPO due to formation of excessive free radicals and failure of antioxidant defense mechanism leading to tissue damage. The phenolic compounds are known to exert protective effect against CCl_4 intoxication by reducing the MDA production, which is indicative of its antioxidant activity.[41] The deficiency of GSH within the living organisms can cause tissue injury and malfunction.[42] In the current study, the liver NP-SH level in CCl_4-treated groups was significantly diminished when compared with the control group. These findings are in accordance with earlier reports as sulfhydryl levels were significantly depleted in different organs of rats, when exposed to CCl_4.[43] Decline in oxidative levels after *A. scrobicularis* administration also indicated the improvement in cellular integrity and status of hepatic cells. Some of related species *Anthemis ruthenica* contains sesquiterpenes and flavonoids are which oxidized by radicals, resulting in a more stable, less reactive radical and flavonoids can also inhibit the activity of many enzymes.[12–17] Histopathological observations after CCl_4-administration showed severe damage in liver and kidney. The prevention of liver and kidney cells texture also account safety and protective nature of ANS extracts in hepatotoxic conditions.

CONCLUSION

The hepatoprotective and nephroprotective activities of ANS were probably due to free radical scavenging properties. The altered hepatic markers such as transaminases, ALP, bilirubin, total protein, MDA and NP-SH with CCl_4 exposure was reversed towards normalization with ANS extract. Bioactive components of ANS probably ameliorated the oxidative damage and had increased the regenerative and restorative ability of liver and kidneys. So it is mandatory to explore the bioactive chemicals in ANS in order to develop the therapeutics that has a promising role in the treatment of hepatotoxic conditions.

CONFLICT OF INTERESTS

There is no conflict of interests.

ACKNOWLEDGMENTS

The authors extend their appreciation to the. Deanship of Scientific Research at Salman Bin Abdulaziz University for the work through the project No. 48-M-1433

REFERENCE

1. Bremer K. *Asteraceae, Cladistics and Classification*. Timber Press Portland Oregon.1994; p.750.
2. Bremer K, Humphries CJ. Generic Monograph of the Asteraceae-Anthemideae. *Bull Nat Hist Mus Lond (Bot.)*. 1993; 23: 71–177.
3. Ghafoor A. The Genus *Anthemis* L. (Compositae-anthemideae) in Arabian Peninsula: A Taxonomic Study. *Pak J Bot*. 2010; 42: 79–98.
4. Amjad L, Madani M, Rezvani Z. Potential Activity of the *Anthemis gayana* Leaves on Fungi. *WASET*. 2012; 61: 901902.
5. Papaioannoua P, Lazaria D, Kariotib A, Soulelesa C, Heilmannc J, Hadjipavlou-Litinad D, Skaltsab H. Phenolic Compounds

with Antioxidant Activity from Anthemistinctoria L. (Asteraceae). *Z Naturforsch*. 2007; 62: 326330.
6. Burim RV, Canalle R, Lopes JLC, Takahashi CS. Genotoxic action of the sesquiterpene lactone glaucolide B on mammalian cells *in vitro* and *in vivo*. *Genet Mol Biol*. 1999; 22: 401–406.
7. Mann C, Staba EJ. *The Chemistry, Pharmacology, and commercial formulations of chamomile. In: Herbs, Spices, and Medicinal Plants: Recent Advances in Botany*, Horticulture and Pharmacology. Craker L. E. and Simon J. E., eds. Oryx Press Phoenix AZ:1986; 1: 235-280.
8. Ugurlu E, Secmen O. Medicinal plants popularly used in the villages of Yunt Mountain (Manisa- Turkey). *Fitoterapia*. 2008; 79:126-131.
9. ManganenelliUncini RE, Tomei PE. Ethnopharmacobotanical studies of the Tuscan Archipelago. *J Ethnopharmacol*. 1999; 65:181–202.
10. Baltaci S, Kolatan HE, Yilmaz O, Kivcak B. Anti-inflammatory activity of *Anthemis aciphylla* var. *aciphylla* Boiss.*Turk J Biol*. 2011; 35:757762.
11. Petkeviciute Z, SavickieneN, Savickas A, Bernatoniene J, Simaitiene Z, Kalveniene Z, PranskunasA, LazauskasR, Mekas TA. Urban ethnobotany study in Samogitia region, Lithuania. *J Med Plants Res*. 2010; 4: 64–71.
12. Konstantinopoulou M, Karioti A, Skaltsas S, Skaltsa H. Sesquiterpene lactones from *Anthemis altissima* and their anti-*helicobacter pylori* activity. *J Nat Prod*.2003; 66: 699-702.
13. Hajdu Z, Zupko I, Rethy B, Forgo P, Hohmann J. Bioactivity-guided isolation of cytotoxic sesquiterpenes and flavonoids from *Anthemis ruthenica*. *Planta Med*. 2010; 76: 94–6.
14. Masterova I, Grancai D, Grancaiova Z, Pour M, Ubik K. A new flavonoid: tinctosid from *Anthemis tinctoria* L. *Pharmazie*. 2005; 60: 956-967.
15. Pavlovic M, Kovacevic N, Tzakou O, Couladis M. Components of cyclohexane extract of Anthemis triumfetti. *Chem Nat Compd*. 2007; 43: 512–514.
16. Vuckovic I, Vujusic L, Milosavljevic S. Phytochemical investigation of *Anthemis cotula*L. *Serb Chem Soc*. 2005; 71: 127-133.
17. Chadwick M, Trewin H, Gawthrop F, Wagstaff C. Sesquiterpenoids Lactones: Benefits to Plants and People. *Int J Mol Sci.* 2013;14: 802–805.
18. Yang W, Luo Y, Aisa HA, Totahon Z, Mao Y, Xu L, Zhang R. Hepatoprotective activities of a sesquiterpine-rich fraction from the aerial part of Cichorium glandulosum. Chinese Medicine. 2012; 7: 1–7
19. Serafini M, Peluso I, Raguzzini A. Flavonoids as anti-inflammatory agents. *Proc Nutr Soc*. 2010; 69: 273–278.
20. Papaioannou P, Lazari D, Karioti A, Souleles C, Heilmann J, Hadjipavlou-Litina D, Skaltsa H. Phenolic compounds with antioxidant activity from *Anthemis tinctoria* L. (Asteraceae).*Z Naturforsch C*. 2007; 62: 326–330.
21. Albayrak S, Aksoy A. Evaluation of Antioxidant and Antimicrobial Activities of Two Endemic Anthemis Species in Turkey. *J Food Biochem*. 2013; 37: 639–645.
22. Rossi T, Melegari M, Bianchi A, Albasini A, Vampa G. Sedative. anti-inflammatory and anti-diuretic effects induced in rats by essential oils of varieties of *Anthemis-nobilis*: A comparative study. *Pharmaceutical Research*. 1988; 20: 71–74.
23. Aithal GP, Day CP. Nonsteroidal anti-inflammatory drug-induced hepatotoxicity. *Clin Liver Dis*. 2007; 11: 563–575.
24. Abere TA, Okoto PE, Agoreyo FO. Antidiarrhoea and toxicological evaluation of the leaf extract of Dissotisrotun difoliatriana (Melastomataceae). *BMC Complement Altern Med*. 2010; 71:1–7.
25. Winter CA, Risley EA, Nuss CW. Carrageenan-induced oedemas in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol Med*. 1962; 111:544–547.
26. Bhadauria M, Nirala SK, Shukla S. Duration-Dependent Hepato-protective Effects of Propolis extract against carbon tetrachloride–induced acute liver damage in rats. *Adv Ther*. 2007; 24:1136–15.
27. Reitman S, Frankel S. A colorimetric method for the determination of serum Glutamic oxaloacetic and glutamic pyruvic transaminases. *Am J ClinPathol.* 1957; 28: 56–63.
28. King EJ, Abul-Fadl MAM, Walker PG. King-Armstrong Phosphatase Estimation by the Determination of Liberated Phosphate. *J ClinPathol*. 1951; 4: 85–91.
29. Szas G. Reaction rate method for gamma glutamyl transferase activity in serum. *Clinical Chem*. 1976; 22: 2031–55.
30. Walters MI, Gerarde HW. An ultra micro method for the determination of conjugated and total bilirubin in serum or plasma. *Microchem J*. 1970; 15: 231–243.
31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement with the Folin Phenol Reagent. *J Biol Chem*. 1951; 193: 265–275.
32. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Annal Biochem*. 1979; 95: 5–8.
33. Sedlak J, Lindsay RH. Estimation of total, protein bound and non-protein SH groups in tissue with Ellman's reagent. *Anal Biochem*. 1968; 25: 192-205.
34. Hajhashemi V, Sadeghi H, Minaiyan M, Movahedian A, Talebi A. Effect of Fluvoxamine on Carrageenan-Induced Paw Edema in Rats Evaluation of the Action Sites. *Iran J Pharm Res*. 2011; 10: 611–618.
35. Jamshidzadeh A, Fereidooni F, Salehi Z, Niknahad H. Hepatoprotective activity of *Gundelia tourenfortii. J Ethnopharmacol*. 2005; 101: 233–237.
36. Zimmerman HJ.Serum enzymes in the diagnosis of hepatic disease. *Gastroenterology*. 1964; 46: 613–618.
37. Wegwu MO, Ayalogu EO, Sule OJ. Anti-oxidant protective effects of *Cassia alata* in rats exposed to carbon tetrachloride. *J Appl Sci Environ*. 2005; 9: 77–80.
38. Yang WJ, Luo YQ, Aisa HA, Xin XL, Totahon Z, Mao Y, Hu MY, Xu L, Zhang RP. Hepato protective activities of a sesquiterpene-rich fraction from the aerial part of Cichorium glandulosum. *Chinese Medicine*. 2012; 7: 1–7.
39. Muriel P, Escobar Y. Kupffer cells are responsible for liver cirrhosis induced by carbon tetrachloride. *J Appl Toxicol*. 2003; 23: 103108.
40. Sies H. Glutathione and its role in cellular functions. *Free RadicBiolMed*. 1999; 27: 916–921.
41. Fan G, Tang JJ, Bhadauria M, Nirala SK, Dai F. Resveratrol ameliorates carbon tetrachloride-induced acute liver injury in mice. *Environ Toxicol Pharmacol*. 2009; 28: 350–356.
42. Ganie SA, Haq E, Hamid A, Qurishi Y, Mahmood Z. Carbon tetrachloride induced kidney and lung tissue damages and antioxidant activities of the aqueous rhizome extract of *Podophyllum hexandrum*. *BMC Complement Altern Med*. 2011; 11: 1–10.
43. Ohta Y, Kongo M, Sasaki E, Nishida K. Ishiguro I. Therapeutic effect of melatonin on carbon tetrachloride-induced acute liver injury in rats. *J Pineal Res.2000*; 28: 119–126.

Anti-diabetic, Hypolipidemic and Anti-oxidant Activities of Hydroethanolic Root Extract of Rhus Mysurensis Heyne in Streptozotocin Induced Diabetes in Wistar Male Rats

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ABSTRACT

Objectives: The present study was designed to investigate the potential role of hydroethanolic root extracts of *Rhus mysurensis* (HERM) in the treatment of diabetes along with its antioxidant and hypolipidemic effects were studied in streptozotocin induced diabetes in Wistar rats. **Methods:** In this study, the anti-diabetic, hypolipidemic and anti-oxidant activities of hydroethanolic root extract of *Rhus mysurensis* was evaluated by using STZ induced diabetic rats at a dose of 200 mg/kg, 400 mg/kg and 800 mg/kg *p.o.* daily for 21 days. Blood glucose levels and body weight were monitored at specific time intervals, and different biochemical parameters, serum cholesterol, serum triglyceride, high density lipoprotein, low density lipoprotein were also assessed in the experimental animals. **Results:** Oral administration of hydroethanolic root extracts of *Rhus mysurensis* (HERM) 400 and 800 mg/kg for 21 days significantly decreased the blood glucose level and considerably increased the body weight of diabetic rats. Daily oral treatment with HERM for 3 weeks resulted in reduced serum cholesterol, and triglycerides and improved HDL-cholesterol levels when compared to the diabetic control group. HERM significantly decreased thiobarbituric acid reactive substances (TBARS) and significantly increased superoxide dismutase and catalase in streptozotocin-induced diabetic rats at the end of 21 days of treatment. **Conclusions:** Hydroethanolic root extract of *Rhus mysurensis* showed significant anti-diabetic, hypolipidemic and anti-oxidant activities, which provide the scientific proof for its traditional claims. The results also put forward that the HERM is promising for development of standardized phytomedicine for the treatment of diabetes mellitus.

Keywords: Anti-diabetic; anti-oxidant; hypolipidemic; *Rhus mysurensis*

INTRODUCTION

Diabetes mellitus (DM) is a most common metabolic disorder of endocrine system. It is characterized by hyperglycaemia resulting from defects in insulin secretion, in insulin action, or both leading to both acute and chronic complications [1,2]. Globally, the incidence of DM is increasing and posing important public health issues.[3] International Diabetes Federation (IDF) estimated the prevalence of DM is approximately 366 million people (8.3%) in 2011 and will rise to 552 million people (9.9%) by 2030. In India, it was around 61.3 million in 2011, rising to 101.2 million by 2030.[4,5] Diabetes mellitus affects 80% of total population of low and middle income countries out of which India and China having larger contribution.[6] It is ninth leading cause of death and encountered around 4.8 million deaths worldwide in 2012. Modern medicines include sulfonylureas, biguanide, thiazolidinedione and α-glycosidase inhibitors and insulin are used for the management of diabetes mellitus.[7] However, these current synthetic drugs are not satisfactory

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DOI: 10.5530/pj.2014.3.10

to cure DM due to less efficacy and several undesirable side effects or contraindications.[8] Therefore, there is a need to develop plant derived anti-diabetic drugs as these are safer, cheaper, and much effective as compare to synthetic drugs. The World Health Organization (WHO) has recommended the screening of medicinal plants for effective treatment of diabetes mellitus and more than 400 plants have been reported till date.[9] Moreover, based on folklore medicine, herbs, spices and several medicinal plants or their extracts have been consumed orally to treat diabetes since ancient times.

Rhus mysurensis Heyne [Family: Anacardiaceae], commonly known as Dansara in Rajasthan.[10] It is a dioecious shrub up to 1.5 m tall and found in rocky areas up to 1500 m. It is distributed in north-western and south-western India i.e. S.E. & W. Punjab, Sind and Rajasthan.[11] It is found abundantly in the New Delhi areas of Aravalli mountain range.[12] It is having spinescent branches, trifoliated leaves with 8–18 mm long petioles, obovate leaflets, terminal panicles or from the axils of the upper leaves, much longer than the leaves. Flowers are unisexual, yellowish. Around 1 mm long stamens, included, abortive in female flowers. Glabrous, ovoid shape ovary found in flower. Distinct styles, each with a capitates stigma. Fruits are yellowish brown, globose, compressed, 4–5 mm broad.[11] Both flowers and leaves are highly scented due to the presence of essential oil. The stem bark of *Rhus mysurensis* is used in tanning industries.[12] Fruits are used for edible purpose in Rajasthan.[10] The root of this plant is used for the treatment of diabetes in Rajasthan based on folklore knowledge. Till date, not even a single study has been performed to explore the pharmacological activity of this plant. The present study was conducted based on the information got from some health practitioners in Sikar district (Rajasthan) of India that decoctions of *R. mysurensis* Heyne roots are effective in the treatment of type 2 Diabetes Mellitus. The present study was undertaken to evaluate the antioxidant, anti-hyperglycaemic and hypolipidemic activity of hydroethanolic root extract of *Rhus mysurensis* Heyne (HERM) in experimental models of streptozotocin (STZ) induced diabetic Wistar rats.

MATERIALS AND METHODS

Drugs and chemicals

The following drugs and chemicals were used in the experiment: glibenclamide and streptozotocin (STZ) were purchased from Sigma-Aldrich, India. Fresh solution of STZ was prepared by dissolving in citrate buffer (0.1 M, pH 4.5). Total cholesterol, high density lipoprotein (HDL) and triglycerides (TC) standard kits were purchased from Merck Specialities Pvt. Ltd, India. All reagents used in this study were of analytical grade.

Plant material

R. mysurensis roots were collected freshly from the hills of Dantaramgarh, Sikar district (Rajasthan), India in July, 2011. Taxonomic identification was done and a voucher specimen was deposited (voucher specimen number RUBL 20605) at the Department of Botany, University Of Rajasthan, Jaipur, India.

Experimental animals

Healthy Male Wistar rats (weighing 200–250 g and age of 3 months) were obtained from the animal house of Gauhati Medical College and Hospital, Guwahati. Animals were housed in polypropylene cages (5 animals per cage), maintained under standard condition (12 h light and 12 h dark cycle; 22–25°C & humidity (60 ± 5%)) and allowed free access to pellet diet and water *ad libitum*. After randomized grouping and before initiation of the experiment, animals were acclimatized to the laboratory conditions. All procedures complied with the guide for the care and use of laboratory Animals and approved by the institutional animal ethics committee, Gauhati Medical College and Hospital, Guwahati.

Extraction

Roots of the plant material was thoroughly washed with distilled water to remove dirt and soil, and dried under shade and optimal ventilation. The plant material was then pulverized and the powdered plant material (700 g) was macerated in water: ethanol (70:30) for 72 h in three successive volumes. The resultant hydro-ethanolic extract was dried under reduced pressure. The extract was evaporated to dryness by warming on a water bath at 60°C and obtained a residue of 64 gm (9.14% yield). The dried extract was kept in a refrigerator until use and used in this study without any further purification.[13]

Preliminary phytochemical screening

Standard screening tests of the extract were carried out for various plant constituents. The crude extract was screened for the presence or absence of secondary metabolites such as reducing sugars, alkaloids, steroidal compounds, phenolic compounds, tannins, saponins, flavonoids, cardiac glycosides, and anthraquinones using standard procedures.[14,15] The results of phytochemical screening of the plant extract are shown in Table 1.

Table 1. Phytochemical screenings of hydro-ethanolic root extract of *Rhus mysurensis*. Where, − = negative; + = weakly positive; ++ = moderately positive; +++ = strongly positive

Chemical constituents	Chemical Tests/reagents	Findings/results
Alkaloids	Dragendorff's reagent/Meyer's reagent	−
Carbohydrates	Molish test	+
Proteins	Biuret test	+
Triterpene steroids	Sulphuric acid reagent	++
Tannins	Ferric chloride reagent	++
Reducing sugar	Fehling's reagent	−
Non-reducing sugar	Iodine solution	+
Flavonoids	Acid-alcohol/solid magnesium/amyl-alcohol	+++
Saponins	Frothing test	++
Anthraquinones	Borntrager's test, BPC	−
Cardiac glycosides	Lieberman's test/Keller-Killiani test	+

Acute toxicity test

Acute toxicity test was done based on the limit test recommendations of OECD 423 Guideline.[16] On day one, Wistar rat fasted for 3–4 h was given 2000 mg/kg of the extract orally. The rat was then kept under strict observation for physical or behavioural changes for 24 h, with special attention during the first 4 h. Following the results from the first rat, other four rats were recruited and fasted for 3–4 h and administered a single dose of 2000 mg/kg and was observed in the same manner. These observations continued for further 14 days for any signs of overt toxicity.

Oral glucose tolerance test (OGTT)

The oral glucose tolerance test[1,17] was performed in overnight fasted (12 h) normal animals. Rats divided into five groups (n = 6) were administered 0.4% CMC solution, hydroethanolic extract (200 mg/kg), hydroethanolic extract (400 mg/kg), hydroethanolic extract (800 mg/kg) and glibenclamide (0.5 mg/kg), respectively. Glucose (2 g/kg) was fed 30 min after the administration of extracts. Blood was withdrawn from the rat tail vein under light ether anaesthesia (to minimize the distress) at 0, 30, 60 and 120 min of extract administration. The fasting blood glucose levels were estimated by glucose oxidase–peroxidase reactive strips (SD check gold, Standard Diagnostics, Inc., Korea).

Induction of diabetes

Diabetes mellitus was induced in 12 h overnight fasted rats by a single intraperitonial injection of freshly prepared STZ at the dose of 40 mg/kg in 0.1 M citrate buffer (pH 4.5).[18] After that animals were left aside for 4 h and then 5% glucose solution was placed in the cages for 24 hours. Diabetes was confirmed after 72 h of STZ administration by checking the blood glucose levels. The mortality within 7 days after STZ injection was found to be 18% and 39 animals out of remaining 50 animals were found diabetic.The diabetic animals were monitored for stabilization of blood glucose level for seven days and study was started on the next day (day 0). Only those animals having blood glucose levels > 200 mg/dl were selected and used for the current study.[19] The body weight and plasma blood glucose levels were measured before and towards end of the experiment.

Experimental design and drug treatment

In the experiment a total of 36 rats (6 normal; 30 STZ diabetic surviving rats) were used. The rats were divided into six groups of six rats each.

Group I: Normal control rats, received a single injection of citrate buffer (vehicle); Group II: Diabetic control rats, received oral gavage of 0.4% CMC once daily for three weeks; Group III: Diabetic rats treated with RM hydroethanolic extract at a dose of 200 mg/kg bw; Group IV: Diabetic rats treated with RM hydroethanolic extract at a dose of 400 mg/kg bw; Group V: Diabetic rats treated with RM hydroethanolic extract at a dose of 800 mg/kg bw; Group VI: Diabetic rats treated with Glibenclamide at a dose of 0.5 mg/kg bw dissolved in 0.4% CMC once daily for three weeks. The extract was dissolved in 0.4% CMC and administered orally in Group III, Group IV, and Group V once daily for three weeks (Figure 1).

At the end of the study, the animals were euthanized between 0900–1100 h to minimize diurnal variation. The changes in body weight and blood glucose levels of all the groups were measured at weekly intervals i.e. 0, 7, 14 and 21 day during the study. Fasting blood glucose level was

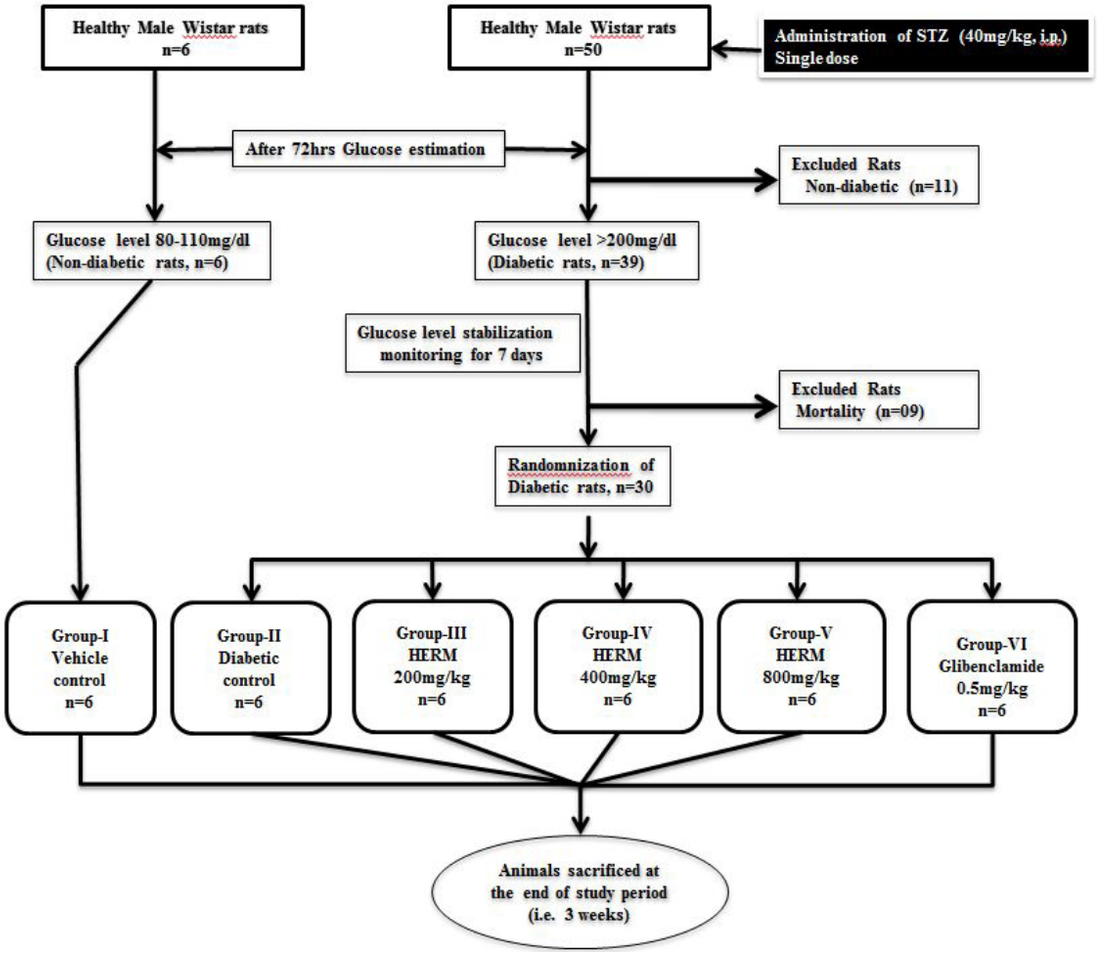

Figure 1. Pictorial representation of the experimental design of the HERM study.

estimated by glucose oxidase – peroxidase method (SD check gold, Standard Diagnostics, Inc., Korea). Finally on day 21, the blood samples were collected through cardiac puncture under mild diethyl ether anesthesia from rats. Then the blood samples were centrifuged at 3000 rpm for 10 min in cold centrifuge at 20°C to obtain serum. Lipid profile [total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride] levels in serum were determined according to the instructions of the manufacturer (Merck, Mumbai, India) with the help of UV-Visible Spectrophotometer (Thermo scientific).[20,21] Low density lipoprotein (LDL) concentration was calculated from the formula of Friedwald *et al.*[22]

Estimation of liver biochemical parameters

Measurement of lipid peroxidation

Liver was homogenized in 2.5% 50 mM PBS buffer pH 7.0 using polytron homogenizer after incubation in triton × 100 for 20 min. Homogenate was used for the measurement of thiobarbituric acid reactive substance at an absorbance of 535nm according to the method of Zhang, 2004.[23]

Measurement of SOD and catalase activity

Liver homogenate was centrifuged at 4°C, 17,500 × g for 10 min, resulted supernatant was used for the measurement of SOD activity by haematoxylin auto oxidation method[24] and catalase activity by hydrogen peroxide (H_2O_2) degradation method.[25]

Statistical analysis

All data were expressed as Mean ± SEM. Between and within group analysis was carried out using one way ANOVA followed by Tukey's post hoc test and level of significance was set at $p < 0.05$. For data processing, Graph Pad Prism data analysis software was used.

RESULTS

Extraction

The percentage yield of ethanolic extract of the dried roots of *Rhus mysurensis* was found to be 9.14% (w/w). The extract was dark-brown semisolid at room temperature and solidified when stored in a refrigerator. Extract returned to semisolid state on re-exposure to room temperature.

Preliminary phytochemical screening

Phytochemical screening of the crude extract of *R. mysurensis* revealed the presence of various secondary metabolites (Table 1). Cardiac glycosides, reducing sugars, steroidal compounds and phenolic compounds, tannins, saponins and flavonoids were detected in the crude extract.

Acute toxicity study

Acute toxicity study of the hydroethanolic extract of *R. mysurensis* did not reveal any behavioral, neurological, autonomic or physical changes such as alertness, motor activity, restlessness, convulsions, coma, diarrhea and lacrimation. Besides, the extract did not cause mortality in the animals at a dose of 2000 mg/kg during the observation time. Thus, the median lethal dose (LD50) of the plant extract is said to be greater than 2000 mg/kg, indicating a good safety margin.

Oral glucose tolerance test in normal rats (OGTT)

Oral glucose tolerance test in normal rats showed that all three doses of HERM i.e. 200, 400 and 800 mg/kg prevented significantly ($P < 0.01$) the increase in blood glucose levels after 30 min of 2 g/kg glucose administration as compared to control. No significant effect was shown by HERM extract at 60 and 120 min. Glibenclamide significantly blocked ($P < 0.01$) the increase in blood glucose levels after glucose administration at 30 min and 60 min (Table 2).

Effect on fasting blood glucose level

After STZ injection, diabetic animals showed significant rise in fasting blood glucose (FBG) level as compared to normal controls. Daily treatment of the extract for prolonged duration (21 days) produced decrease in blood glucose levels in diabetic rats in a dose dependent manner. These fall in FBG were significant (P<0.01) when compared to diabetic control. Blood glucose level at 21st days was (213.67 ± 6.51), (197.67 ± 14.68) and (136.17 ± 8.40) mg/dL at the doses of 200, 400 and 800 mg/kg of HERM respectively. At the end of study (on 21st day) HERM extract at 800 mg/kg BW treated group decreased the FBG level significantly (53.33%) as compared to diabetic control. The effects of HERM extract on the FBG level of normal and diabetic animals is also shown in Table 3.

Effect on body weight

The body weight change of experimental animals during study is shown in Table 4. There was significant reduction in body weight of diabetic control rats as compared to normal control rats. At the termination of study (on 21st day), there was significant (P<0.01) increase in body weight in HERM treated group in dose dependent fashion and in glibeclamide treated group when compared with diabetic control rats.

Effect on lipid profile

Oral administration of HERM showed dose dependent hypolipidemic activity. It reduced plasma cholesterol, triglyceride, LDL in STZ treated rats. In addition, to hypolipidemic activity, HERM also produced a noteworthy dose dependent increase in level of high density lipoproteins (HDL). High density lipoprotein (HDL) is commonly referred to as good cholesterol possessing the ability to reverse cholesterol transport and also protect LDL from oxidation, thereby minimizing the deleterious consequences of LDL oxidation. HERM 800mg/kg showed highest decrease in the level of plasma cholesterol, triglyceride, LDL whereas increase in HDL level in STZ treated rats as compared to the left over groups of HERM (Table 5).

Effect on Oxidative parameters

The levels of TBARS were significantly (P<0.001) increased in STZ control animals as compared to normal control group. Treatment with HERM 400mg/kg (P<0.001) and 800 mg/kg (P<0.001) significantly reduced the TBARS levels when compared with STZ control animals in dose related manner (Figure 2). The level of SOD was significantly (P<0.001) depleted in STZ control group as compared with normal control group. Reduced SOD level was found to be dose dependently elevated towards normal level upon administration of HERM as compared with STZ control group. Moreover, HERM 400 and 800 mg/kg showed statistically significant elevated levels of SOD when compared with STZ control group (Figure 3). There was significant (P<0.001) reduction in

Table 2. Effect of hydroethanolic root extract of *R. mysurensis* on oral glucose tolerance test. All the values were expressed as mean ± SEM (n = 6); ***P< 0.001. a vs control

Groups	Oral Glucose Tolerance Test			
	0 min	30 min	60 min	120 min
Control	75.1 ± 3.17	108.3 ± 2.15	101.3 ± 1.83	82.0 ± 2.65
Diabetic Control	71.2 ± 3.21	84.4 ± 2.1^{0***}a	96.6 ± 2.71	80.3 ± 2.46
HERM (200mg/kg)	73.5 ± 3.14	89.5 ± 2.2^{1***}a	97.5 ± 2.87	79.6 ± 2.27
HERM (400mg/kg)	78.3 ± 4.10	93.3 ± 1.99^{***a}	95.0 ± 2.58	82.3 ± 2.27
HERM (800mg/kg)	73.1 ± 3.30	82.1 ± 2.81^{***a}	78.6 ± 2.2^{7***}a	74.6 ± 2.33

Table 3. Effect of hydroethanolic root extract of *R. mysurensis* on fasting blood glucose level in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); ***P< 0.001, *P<0.05. a vs control & b vs Diabetic control

Groups	Fasting Blood Glucose Level (g/dl)			
	Day 0	Day 7	Day 14	Day 21
Control	76.33 ± 4.60	77.0 ± 4.37	75.3 ± 3.83	77.5 ± 4.35
Diabetic Control	295.6 ± 15.0^{4***}a	308.8 ± 14.0^{5***}a	313.3 ± 14.2^{2***}a	320.6 ± 14.1^{9***}a
HERM (200mg/kg)	287.8 ± 11.56	266.8 ± 11.54	241.1 ± 9.8^{5***}b	213.6 ± 6.5^{1***}b
HERM (400mg/kg)	311.5 ± 13.07	256.8 ± 13.7^{6*}b	220.1 ± 13.6^{3***}b	197.6 ± 14.6^{8***}b
HERM (800mg/kg)	291.8 ± 13.06	225.3 ± 11.7^{2***}b	174.6 ± 10.7^{0***}b	136.1 ± 8.4^{0***}b
Glibenclamide (0.5mg/kg)	291.8 ± 13.06	203.0 ± 12.10	160.3 ± 9.64	120.6 ± 7.37

Table 4. Effect of hydroethanolic root extract of *R. mysurensis* on body weights in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); ***P< 0.001, **P<0.001, *P<0.05. a vs control & b vs Diabetic control

Groups	Body Weight (g)			
	Day 0	Day 7	Day 14	Day 21
Control	230.6 ± 3.94	234.6 ± 4.63	239.3 ± 4.55	243.2 ± 3.56
Diabetic Control	230.3 ± 5.01	214.1 ± 4.7^{1*}a	203.5 ± 4.1^{3***}a	197.1 ± 4.0^{0***}a
HERM (200mg/kg)	224.0 ± 4.18	223.0 ± 4.04	227.1 ± 4.4^{3*}b	230.8 ± 4.0^{6***}b
HERM (400mg/kg)	225.1 ± 4.87	221.0 ± 4.97	228.1 ± 4.72^{**b}	234.3 ± 5.4^{2***}b
HERM (800mg/kg)	221.3 ± 4.34	220.1 ± 4.20	229.6 ± 5.4^{4**}b	236.8 ± 5.6^{6***}b
Glibenclamide (0.5mg/kg)	227.6 ± 3.90	226.0 ± 3.96	234.6 ± 4.2^{5***}b	240.5 ± 4.6^{9***}b

Table 5. Effect of hydroethanolic root extract of *R. mysurensis* on serum lipid profile in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); ***P< 0.001, **P<0.001, *P<0.05. a vs control & b vs Diabetic control. CH=Cholesterol, TGs=Triglycerides, HDL=High density lipoproteins, LDL=Low density lipoproteins

Groups	Total-CH (mg/dl)	TGs (mg/dl)	HDL-CH (mg/dl)	LDL-CH (mg/dl)
Control	79.72 ± 3.06	81.02 ± 3.80	34.05 ± 1.70	29.48 ± 3.78
Diabetic Control	168.69 ± 6.0^{0***}a	140.95 ± 5.8^{8***}a	19.53 ± 2.2^{5***}a	120.97 ± 5.9^{2***}a
HERM (200mg/kg)	132.88 ± 3.8^{1***}b	120.39 ± 5.2^{7*}b	23.13 ± 1.46	85.68 ± 4.8^{8***}b
HERM (400mg/kg)	116.21 ± 2.9^{6***}b	113.47 ± 4.2^{4**}b	25.85 ± 1.51	67.67 ± 3.6^{4***}b
HERM (800mg/kg)	91.82 ± 3.0^{6***}b	99.22 ± 3.4^{4***}b	29.61 ± 2.2^{3*}b	42.24 ± 3.8^{7***}b
Glibenclamide (0.5mg/kg)	87.46 ± 4.68	101.77 ± 3.55	29.32 ± 2.45	37.81 ± 6.81

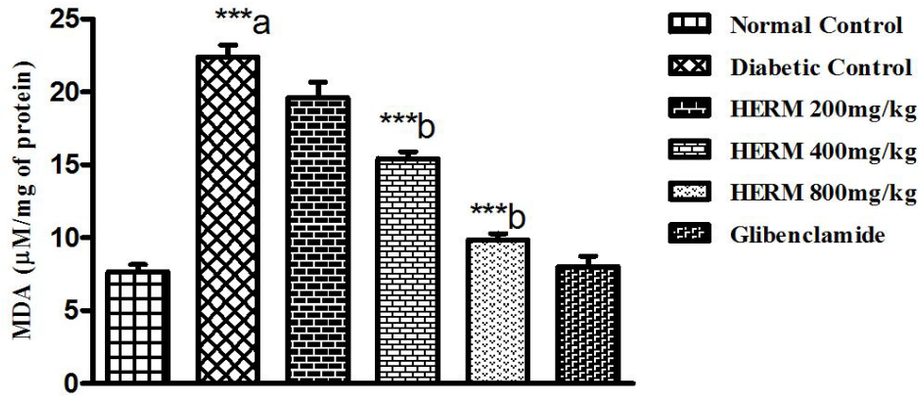

Figure 2. Effect of hydroethanolic root extract of *R. mysurensis* on MDA level in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); a vs Normal control, b vs Diabetic control. ***P<0.001.

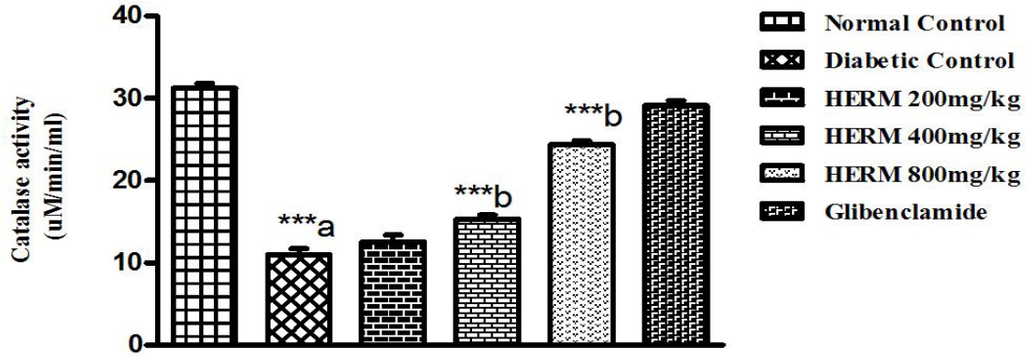

Figure 3. Effect of hydroethanolic root extract of *R. mysurensis* on SOD activity in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); a vs Normal control, b vs Diabetic control. ***P< 0.001.

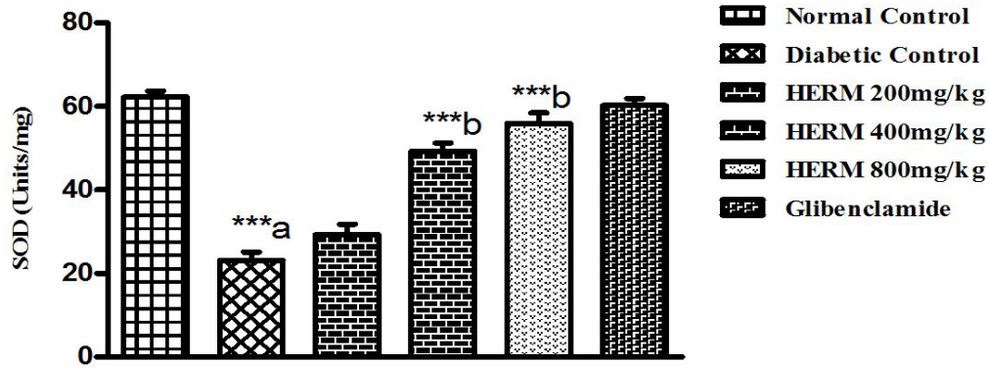

Figure 4. Effect of hydroethanolic root extract of *R. mysurensis* on catalase activity in STZ induced diabetic rats. All the values were expressed as mean ± SEM (n = 6); a vs Normal control, b vs Diabetic control. ***P< 0.001.

catalase activity in STZ control group compared with normal group. The administration of 400 and 800mg/kg doses of HERM recovered CAT activity significantly (P<0.001) towards normal when compared with STZ control animals (Figure 4).

DISCUSSION

The present study for the first time reports the antioxidant, antihyperglycemic and hypolipidemic activities of HERM in STZ-induced diabetic Wistar rats. The results of this

study revealed that HERM at doses of 400 and 800 mg/kg significantly normalized elevated blood glucose level and restored serum and liver biochemical parameters towards normal values. Streptozotocin (STZ) is a nitrosourea compound produced by *Streptomyces achromogenes*, which specifically induces DNA strand breakage in β-cells causing diabetes mellitus. This leads to insulin deficiency which in turn increases the blood sugar level[9]. The fundamental mechanism underlying hyperglycemia involves over-production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilization of glucose by the tissues.[26] Persistent hyperglycemia, the common characteristic of diabetes can cause most diabetic complications. In all patients, treatment should aim to lower blood glucose to near-normal levels.[27]

Hyperglycemia was observed after 48 hours of STZ induction. Treatment with HERM in STZ-induced diabetic rats started reducing fasting blood glucose levels in a dose dependent manner after 7 days and made them normoglycemic after 21 days. The antihyperglycemic effect of HERM at a dose of 800 mg/kg was found to be comparable to the effect exerted by the reference drug glibenclamide at a dose of 0.5 mg/kg. Normal healthy animals were found to be stable in their body weight whereas diabetic animals showed reduction in body weight. The losses in weights of diabetic animals were due to the increased muscle wasting and loss of tissue proteins. In this study, the reduction of body weight was diminished by extracts after 21 days of treatment in a dose dependent manner.

Since lipid abnormalities accompanying with premature atherosclerosis is the major cause of cardiovascular diseases in diabetic patients, therefore ideal treatment for diabetes, in addition to glycaemic control, should have a favourable effect on lipid profile. Cardiovascular diseases are listed as the cause of death in 65% people suffering from diabetes.[28-31] From this point of view, it is encouraging that a regular administration of HERM extract for 21 days nearly normalized lipid profile in diabetic animals. The dose of 800 mg/kg not only lowered TC, TG and LDL but also enhanced the cardio-protective lipid HDL. The LD50 of the extract is high (no death even with 15 times of effective dose) indicating high margin of safety. The fall of 50 and 75% in plasma sugar of severely diabetic group after 7 and 14 days of treatment of most effective dose further confirms our findings.

Oxidative stress in diabetes mellitus has been shown to coexist with impairment in the endogenous antioxidant status.[32] Our study results showed that HERM strongly restored liver antioxidant parameters and decreased lipid peroxidation in diabetic animals. The reduction in liver antioxidant status during diabetes may be the result of counteraction against increased formation of lipid peroxides.[33] A conspicuous increase in the concentration of TBARS in STZ-induced diabetic rats indicated greater lipid peroxidation leading to tissue injury and failure of the endogenous antioxidant defence mechanisms to prevent overproduction of free radicals. Lipid peroxidation is generally measured in terms of TBARS as a biomarker of oxidative stress.[34] Treatment with HERM for 21 days inhibited hepatic lipid peroxidation in diabetic rats as shown by the reduction of TBARS levels towards normal levels, suggesting that HERM could improve the pathologic condition of diabetes by inhibiting lipid peroxidation in STZ treated rats.

Enzymatic antioxidant mechanisms play an important role in the elimination of free radicals (ROS).[35] A reduction in the activities of these enzymes results in the accumulation of superoxide anion and hydrogen peroxides which would have otherwise been effectively scavenged by these enzymes. HERM treatment for 21 days significantly recovered the hepatic SOD and CAT activities towards normal in a dose dependent manner, indicating a protective role of the extract. This may be attributed to the presence of phytochemicals such as phenol and flavonoids. This is further supported by evidence indicating the use of natural extracts from plant source in reducing the risk of oxidative stress, due to their rich source of phytochemicals.

Phytochemical investigation of HERM reveals the presences of sterols, saponins, coumarins, quinones, tannins, flavanoids. These principles are documented to be bioactive for the management of diabetes. It is well known that certain flavonoids exhibit hypoglycemic activity and pancreas beta cell regeneration ability. Sterols have also shown to decrease blood sugar in experimental animal models.[21] Thus, the significant antidiabetic HERM may be due to the presence of more than one antihyperglycemic principle and their synergistic properties.

In the present study, the administration of HERM to STZ-induced hyperglycemic rats demonstrated prominent reduction in blood sugar level, normalization of biochemical profile including lipid contents, as compared to diabetic control rats. Moreover, HERM treatment resulted in significant modulation of lipid peroxidation, endogenous enzymatic antioxidant and detoxification status. Hence, it can be concluded that the hydroethanolic extract of *R. mysurensis* root is remarkably effective against streptozotocin-induced diabetes in Wistar rats

possibly by virtue of its augmenting the endogenous antioxidant mechanisms. Further pharmacological and biochemical investigations should be done to elucidate the mechanism of the antidiabetic and hypolipidemic effect of *R. mysurensis*.

CONCLUSION

In conclusion, the present study demonstrates that HERM (400 and 800 mg/kg, *p.o.*) at the dose levels tested reveals potent antidiabetic, hypolipidemic and antioxidant activities in STZ induced diabetes in male Wistar rats and also shows the improvement in oral glucose tolerance in glucose-loaded normal rats without inducing hypoglycemic state. The agent with these multiple beneficial effects viz., antidiabetic, hypolipidemic, and antioxidant properties without causing hypoglycemia would be of greater therapeutic benefit in the management of diabetes associated with abnormalities in lipid profiles and merits further detailed investigation to find out its mechanism of action and to establish its therapeutic potential in the treatment of diabetes and diabetic complications.

ACKNOWLEDGMENT

This work was supported by a grant from the National Institute of Pharmaceutical Education and Research, Guwahati, India. We sincerely acknowledge Prof. K C Saikia, Principal, GMC, Guwahati for providing necessary facilities to carry out this work.

REFERENCES

1. Yadav JP, Saini S, Kalia AN, Dangi AS. Hypoglycemic and hypolipidemic activity of ethanolic extract of Salvadora oleoides in normal and alloxan-induced diabetic rats. Indian J Pharmacol. 2008; 401:23–27.
2. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2010; 33 Suppl 1:S62–69.
3. Tabish SA. Is Diabetes Becoming the Biggest Epidemic of the Twenty-first Century? Int J Health Sci (Qassim), 2007; 12:V–VIII.
4. Federation ID. International Diabetes Federation IDF Diabetes Atlas, Brussels, Belgium, 2012; 5th edn.: http://www.idf.org/diabetesatlas.
5. Healthmeup. Type 2 Diabetes: Shocking Diabetes Facts. 2013; http://healthmeup.com/news-healthy-living/type-2-diabetes-shocking-diabetes-facts/20850.Last accesed on 20/08/2013.
6. Ramachandran A, Das AK, Joshi SR *et al.* Current Status of Diabetes in India and Need for Novel Therapeutic Agents. JAPI. 2010; 58:7–9.
7. Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. Oman Med J.2012; 274:269–73.
8. Balamurugan R, Ignacimuthu S. Antidiabetic and Hypolipidemic effect of methanol extract of Lippia nodiflora L. in streptozotocin induced diabetic rats. Asian Pacific Journal of Tropical Biomedicine. 2009:S30–S36.
9. Kumar S, Kumar V, Prakash O. Antidiabetic and hypolipidemic activities of Kigelia pinnata flowers extract in streptozotocin induced diabetic rats. Asian Pac J Trop Biomed. 2012; 27: 543–46.
10. Kotia A, Kumar A. Characterization of biodiversity of Indian desert and its evaluation. Indian Journal of Plant Sciences. 2012; 1:2–3.
11. Nasir YJ. Rhus in Flora of Pakistan. Science Press and Botanical Garden Press: Beijing; Missouri. 2013.
12. Srivastava S, Mallavarapu GR, Rai SK *et al.* Composition of the essential oils of the leaves and flowers of Rhus mysurensis Heyne ex Wight & Arn growing in the Aravalli mountain range at New Delhi. Flavour Fragr J. 2006; 21:228–29.
13. Gupta AK. Introduction to pharmaceutics-1. CBS Publication. 1994; 3rd Edi 11:P. 147.
14. Kokate CK, Purohit AP, Gokhale SB. Text book of Pharmacognosy. 26 Edition. Nirali Prakashan: Pune. 2006.
15. Khandelwal KR. Practical Pharmacognosy. 16 Edition. Nirali Prakashan: Pune. 2006.
16. OECD. Guidelines No. 423 for testing of chemicals revised draft guideline 423 (acute oral toxicity) class methods. 2001.
17. Bonner-Weir S. Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans. Diabetes. 1988; 375: 616–21.
18. Orhan N, Aslan M, Orhan DD, Ergun F, Yesilada E. In-vivo assessment of antidiabetic and antioxidant activities of grapevine leaves (Vitis vinifera) in diabetic rats. J Ethnopharmacol. 2006; 1082:280–86.
19. Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Bodhankar SL. Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy. Fitoterapia. 2012; 834:650–59.
20. Sachdewa A, Khemani LD. Effect of Hibiscus rosa sinensis Linn. ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. J Ethnopharmacol. 2003; 891:61–66.
21. Tietz. Textbook of Clinical Chemistry. 3rd Edition. W.B. Saunders Company: Philadelphia .1999.
22. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972; 186:499–502.
23. Zhang DL, Zhang YT, Yin JJ, Zhao BL. Oral administration of Crataegus flavonoids protects against ischemia/reperfusion brain damage in gerbils. J Neurochem. 2004; 901: 211–19.
24. Martin JP, Jr. Assays for superoxide dismutase based on autoxidation of hematoxylin. Methods Enzymol. 1990; 186:220–27.
25. Stevens MJ, Obrosova I, Cao X, Van Huysen C, Greene DA. Effects of DL-alpha-lipoic acid on peripheral nerve conduction, blood flow, energy metabolism, and oxidative stress in experimental diabetic neuropathy. Diabetes. 2000; 496: 1006–1015.
26. Kalaivanan K, Pugalendi KV. Antihyperglycemic effect of the alcoholic seed extract of Swietenia macrophylla on streptozotocin-diabetic rats. Pharmacognosy Res. 2011; 31:67–71.
27. Bloomgarden ZT. American Diabetes Association Annual Meeting, 1998: cardiac disease and related topics. Diabetes Care.1998; 21 10:1764–1773.
28. Grundy SM, Benjamin IJ, Burke GL *et al.* Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. Circulation. 1999; 100 10:1134–1146.
29. Kesari AN, Kesari S, Singh SK, Gupta RK, Watal G. Studies on the glycemic and lipidemic effect of Murraya koenigii in experimental animals. J Ethnopharmacol. 2007; 112 (2):305–11.

30. Patil RN, Patil RY, Ahirwar B, Ahirwar D. Evaluation of antidiabetic and related actions of some Indian medicinal plants in diabetic rats. Asian Pac J Trop Med. 2011;41:20–23.
31. Kumar S, Kumar V, Prakash O. Antidiabetic, hypolipidemic and histopathological analysis of Dillenia indica (L.) leaves extract on alloxan induced diabetic rats. Asian Pac J Trop Med. 2011; 45:347–52.
32. Gomathi D, Ravikumar G, Kalaiselvi M, Devaki K, Uma C. Efficacy of Evolvulus alsinoides (L.) L. on insulin and antioxidants activity in pancreas of streptozotocin induced diabetic rats. J Diabetes Metab Disord. 2013; 121:39.
33. Gokce G, Haznedaroglu MZ. Evaluation of antidiabetic, antioxidant and vasoprotective effects of Posidonia oceanica extract. J Ethnopharmacol. 2008; 1151:122–30.
34. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med. 1990; 96:515–40.
35. Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ Med J. 2012; 121:5–18.

Pharmacognostical studies on the fruit of *Elaeocarpus oblongus* Gaertn.

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ABSTRACT

Elaeocarpus tectorius (Lour.) Poir, Synonym: *Elaeocarpus oblongus* auct. non Gaertn. Elaeocarpaceae, is a tree, found throughout Western Ghats, South India. The present study indented to establish the pharmacognostical and physicochemical quality control parameters of *E.oblongus* fruits to avoid confusion in taxonomic identification. Physicochemical evaluation of fruit was carried out according to the guidelines of WHO/QCMMP and Indian Ayurvedic Pharmacopoeia. The elemental analysis was done by using Perkin Elmer 5000 an atomic absorption spectrophotometer. Non glandular unicellular trichomes found to be distinguished character of powder analysis. It was quantified to be 700μm long and 400μm thick at the base. Lerachysclereids were found plenty in powder. The rosettes type of calcium oxalate crystals were 15 μm in diameter. Cells of the endosperm showing darkly stained globular bodies and the cotyledon is 170μm thick. Physio-chemical parameters such as total ash and acid-insoluble ash (2.66% w/w, 0.66% w/w, respectively), extractive values (aqueous 31.068% w/w and alcoholic 30.94% w/w), foreign organic matter (0.5% w/w) and loss on drying (12% w/w) were estimated. Qualitative analysis showed the presence of Fructose, Glucose, Flavanoids and Tannins and Sterols and Phenolic compounds and fatty acids in the fruit. The quantity of elements (μg/g) in the fruit pulp powder was estimated by elemental analysis. The result shows Mn-53.5 and Zn – 46.2 were the major contents. While Pd- 14.3, Cu- 7.5 and Cr- 4.9 were minor contents. This study provided the pharmacognostical profile used to differentiate the other similar looking fruit from other ones of this genus.

Keywords: Western Ghats, Budagas, Ooty, Quality control, Elaeocarpaceae, Edible fruit

INTRODUCTION

Elaeocarpus is a genus of tropical and subtropical trees distributed from Madagascar in the west through India, Southeast Asia, Malaysia, Japan, and Australia to New Zealand, Fiji and Hawaii in the east with its estimated about 350 species.[1] In India most of the species grow in the Himalayan region.[2] *Elaeocarpus* species possess many biologically active molecules they are Indolizilidine alkaloids, Triterpenes, Tannin such as Geraniin and 3, 4, 5-trimethoxy geraniin, Grandisines, Rudrakine and Flavonoids; Quercitin. Most of the *Elaeocarpus* species have exhibited Anti-inflammatory, Antimicrobial, Anti anxiety and Analgesic, Antidepressant and Antihypertensive activities.[3] The ancient literature shows *Elaeocarpus* was used as an Ayurvedic medicine, but biological studies revealed that a number of other therapeutic uses of this genus make known its species as the chief source of multi-use medicinal agent proved in experimental animals.[4]

Elaeocarpus tectorius (Lour.) Poir, Synonym: *Elaeocarpus oblongus* auct. non Gaertn., Elaeocarpaceae,[5] is found wild in Western Ghats ascending to 6,000 ft.[6] The tribe of Nilgiris district todas, kurumbas, kothas, irulas, kattanaikes are broadly using these fruits for therapeutic purpose. During our field trip, we had seen that the fruits were very cheapest resource for the treatment of rheumatism and body pain. It has been reported further various

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DOI: 10.5530/pj.2014.3.11

ancient tribes were using this fruit for the treatment of leprosy, pneumonia, rheumatism, ulcers, piles, and dropsy.[7–9] Literature shows very few biological studies had been conducted on leaf, stem and stem bark. But there was no report on pharmacognostic evaluation of much used part of the tree the fruit. Since the fruits are edible and important seasonal food commodity (May to August) and it is extensively sold in the local market of Nilgiris district, Tamilnadu state, India. The current study, has decided to investigate the anatomical, physicochemical characters and the presence of sugars in edible fruits of *Elaeocarpus oblongus*. This is the first time the fruit was assessed for anatomical and physicochemical characters.

MATERIALS AND METHODS

Study area

Udhagamandalam commonly known as Ooty, is the administrative capital of Nilgiris district. The district, as the Blue Mountain is a tourist resort and populated by the tribes of Badagas and Todas, Irulas, Panias, Kurumbas. Ooty is spread out at 11.24 degree North latitude and 76.41 degrees of longitude.[10] The queen of hill-Nilgiris[11] is made the south boundary of the Western Ghats structure that extended continuously from Mumbai in the north to the Nilgiris in the south. The altitude of this region ranges from 1700 to 2400 m above the sea level. The annual average rainfall is 1590 mm.The annual temperature ranges from 4°C to 24°C.The monsoon starts from June to October. The lateritic, dark brown, loamy soil is present in the study area.[12]

Collection of specimens

The fresh fruits and plant specimen were collected early morning during the summer season in the month of May 2012 from the "Bikki" tree at the Edakadu village near to the Ooty hill station, South India. The plant material was taxonomically identified by Prof.P. Jayaraman, Taxonomist, Plant Anatomy Research Centre, Chennai. The Voucher specimen (No.PARC/2010/2098) was deposited in the medicinal plant documentation unit in Pharmacognosy and Phyto chemistry department, Nehru College of Pharmacy, Pampady, Thiruvilwamala-680597, Thrissur district, Kerala state, India. Fresh fruits were used for microscopic characterization while dried fruit pulp powder has been used to determine the physicochemical parameters such as ash values and extractive values and powder microscopic characters and qualitative and fluorescence analysis. Reagents, Chemicals of analytical grade were used from Sigma Chemical Co, St. Louis, USA and Fine Chemicals Ltd., Mumbai, India.

Microscopic slide preparation

Fruit was fixed in FAA (Formalin-5 ml + Acetic acid-5 ml + 70% Alcohol-90 ml). Later on microscopic slides were prepared according to the usual plant anatomy protocols.[13-14] The transverse cross sections of the fruit were obtained by cutting the paraffin embedded[15-16] specimen by Rotary Microtome. The thickness of the sections was measured about 10–12 µm. De waxing of the sections was done by standard procedure,[14] subsequently the sections were stained with toluidine blue.[17] Sections of the fruit were cleared in 30% sodium hypochlorite solution and stained with safranin.[14] Microscopic characterization of fruit was done by Olympus optical microscope attached to a digital camera Sony. The powder microscopy was done by standard procedures [18,19] and micro chemical tests for histological region were performed according to the standard methods.[15&20–22] An anatomical feature of the description was made with the help of the standard anatomy book.[23]

Behavior of powder with different chemical reagents

The powder material was treated with different chemical reagents to detect the phytoconstituents by observing colour changes under ordinary daylight and also the colour and consistency of extracts were also observed by the standard method.[24]

Fluorescence analysis of powder and extracts

The fruit extracts were examined and analysed in daylight, short and long UV light for fluorescence, according to the standard methods.[25]

Estimation of inorganic constituents

To estimate the inorganic elements contents, 1 g of the fruit pulp, dried powder was digested with concentrated nitric acid and perchloric acid (3:1) until a clear solution was obtained. Subsequent to cooling, the solution was made up to a specific volume with the de mineralized water and analysed in Perkin Elmer 5000 an atomic absorption spectrophotometer.[26]

Physicochemical parameters

Loss on drying,[27] pH of 1% aqueous soluble portion of the fruit was calculated at 25°C,[28] Ash values and extractive values were carried out based on the standard protocol.[19] Thin Layer Chromatography (TLC) was performed for fruit extracts according to the standard procedure.[29,30]

Determination of fruit sugar and vitamins

Qualitative analysis of fruit powder was carried out by using the standard procedure to determine the Sugars.[31]

RESULTS

The presence of Inorganic elements and specified physicochemical parameters were determined (Table-1). The TLC Profile of successive solvent extracts is a fundamental module of the contemporary monograph was depicted in table 2. The taste of the fresh fruit was sugary. The Qualitative chemical test report was shown the presence of Glucose, Fructose were the sources of carbohydrate present in the fruit. The secondary metabolites such as tannin, phenolic compounds and flavonoids, and sterols are the secondary metabolites in the fruit. The behaviors of powder with different chemical reagents are depicted in table 3. In fluorescence analysis, Fruit pulp powder was treated with different alkalis and acids had undergone the short and long UV examinations. The results show 1NHCl given pale yellow and brownish yellow and 1N NaOH produced yellow, brown and yellowish green and 50% HNO_3 turned green and greenish brown, 1N NAOH in Methanol produced dark brown and brownish green and 50% H_2SO_4 given pale reddish and reddish brown and Methanol in nitrocellulose turned yellow and dark green under short and long UV respectively.

Table1. Physicochemical values of fruit of *Elaeocarpus oblongus*

Parameters	Results
1. Organoleptic characteristics.	
Appearance	Coarse powder
Colour	Greenish white
Odour	Characteristic
2. Loss on drying	12%w/v
3. pH values	
pH of 1% aqueous solution	5.2
4. Ash values (%)	
Total ash	2.66%
Acid insoluble ash	0.66%
Sulphated ash	3.033%
Water soluble matter (%)	31.068%
Alcohol soluble matter (%)	30.94%
5. Successive solvent extractives (%)	
Hexane extract	0.23%
Chloroform extract	0.25%
Ethyl acetate extract	0.47%
Ethanol extract	15.66%
Aqeous extract	10.40%
6. Foreign organic matter	0.5% w/w
7. Elemental analysis	
In organic Elements	Quantity of elements (µg/g) in dried powder
Zn	46.2
Mn	53.3
Cu	7.5
Cr	4.9
Pb	14.3

Studies on Microscopic characters of *Elaeocarpus oblongus*

The fruit is a drupe with stony tuberculate pericarp. The seeds have a hard seed coat. The pericarp has a thin epidermal layer of small cells which are thick walled. The mesocarp very wide, comprises entire cells being thick walled, homogeneous and the cells have tannin contents (Figure 1). When the fruit matures the cells of mesocarp become thick walled and lignified sclerides scattered in the mesocarp are small clusters of vascular bundles which comprise a few xylem and phloem elements (Figure 2.1).

In the young fruit small clusters of lerachyscelerides are seen sparingly distributed in the mesocarp (Figure 2.2). The ground parenchyma cells of the mesocarp are polygonal in the outer zone (Figure 1) and the cells become gradually oblong in the inner zone.

Endosperm

Figure 3.3 depicted the endosperm tissues are thick towards the micropylar end and gradually become thinner towards the chalaza end (Figure 2.3). The endosperm is 1.7 mm thicker at the micropylar end and less than 1mm through the opposite part. The endosperm has fairly thick epidermis comprising very vertically oblong cells. The cells inner in the epidermis are squarish, thin walled and compact towards the inner part of the endosperm, the cells get arranged in regular, compact parallel lines. All the cells possess some darkly stained spherical bodies of varying sizes; the chemical nature of these bodies not known.

Embryo

The embryo consists of flat, uniformly thick cotyledons. It consists of inner and outer, fairly distinct epidermal layers and about 8 layers of squarish compact parenchyma cells of ground tissue. The cells contain large, darkly stained bodies. The cotyledon is 170µm thick.

Table 2. TLC finger printing of different extracts of fruit of *Elaeocarpus oblongus*

Extract	Solvent system	Detection	Spots	hR_f Values
Hexane	Toluene: methanol (25:75)	Ceric sulphate- Sulphuric acid (65%)	5	04;34;52;58; 65
Chloroform	n-hexanae Acetone (20:80)	Ceric sulphate- Sulphuric acid (65%)	3	06; 16;20
Ethyl acetate	Toluene : Methanol (80:60)	Ceric sulphate- Sulphuric acid (65%)	3	12;22; 39
Ethanol	CH_2CL_2 Methanol (20:80)	Ceric sulphate- Sulphuric acid (65%)	4	06; 36;41;54

Table 3. Behavior of the powder with different chemical reagents of fruit of *Elaeocarpus oblongus*

Reagents	Observation	Chemical nature
Aqeous $Fecl_3$	Green colour	Tannins/Flavanoids Present
Dilute ammonia solution	No change	Anthraquinone absent
5%Aqeous KOH	No change	Anthraquinone absent
Aqeous $Hgcl_2$	No precipitate	Alkaloids absent
Picric acid	No precipitate	Alkaloids absent
Aqeous $AgNo_3$	No white precipitate	Protein absent
Conc H_2So_4	Reddish brown	Steroids/Triterpenoids present
Mg-Hcl	Magenta Yellow	Flavanoids present
Picric acid	No change	Alkaloids absent
Iodine solution	No change	Starch absent
Mayer's reagent	No precipitate	Alkaloids absent
Aqueous Lead aceate	white precipitate	Tannins present
Aqueous NAOH	Yellow colour	Flavanoids present

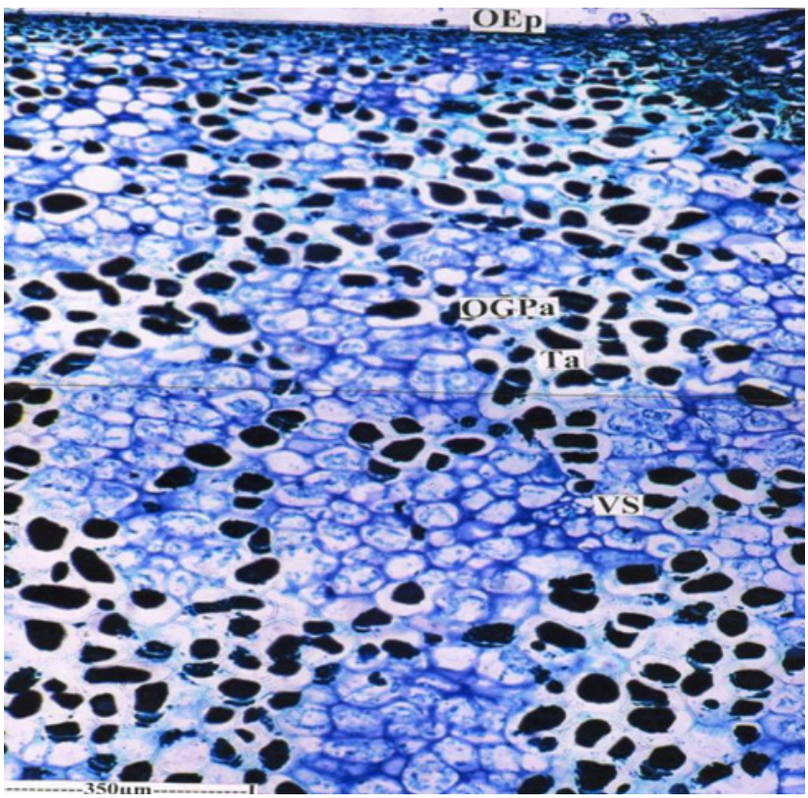

Figure 1. T.S. of Pericarp of the young fruit showing vascular strands and abundance of tanniferous cells. OEP – Outer epidermis, OGPA – Outer ground parenchyma, Ta- Tannin, VS- Vascular strand.

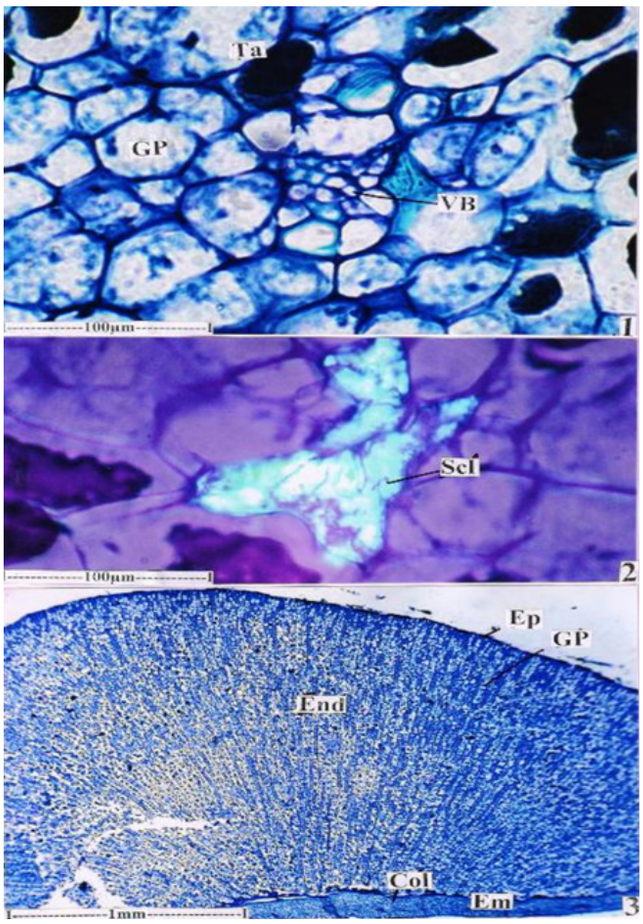

Fig. 2.1 A vascular strand of the pericarp enlarged.
2.2 A small cluster of sclereids in the pericarp (polarized microscopy).
2.3 T.S. of cotyledon.
End – Endosperm, Em – Embryo, EP – Epidermis, GP – Ground parenchyma, Scl – sclereids, Ta – Tannin, VB – Vascular bundle

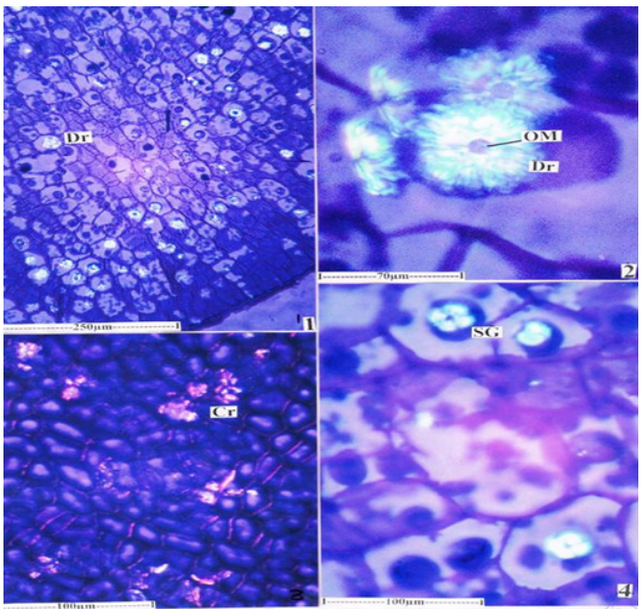

Fig 3.1 distribution of crystals in the endosperm cell.
3.2 A single rosette type of crystals.
3.3 Crystals in the pericarp of the fruits.
3.4 Starch grains in the endosperm.
Cr – crystal, Dr – druses, OM – Organic matter, SG – starch grain.

Crystal distribution

Calcium oxalate crystals fairly common in the endosperm cells (Figure 3.1, 3.2) and in the sclerotic mesocarp of the fruit (Figure 3.3). In the endosperm cells the crystals are rosette type, they are circular bodies with central dark organic matter (Figure 3.1, 3.2) some druses are also seen in the cells. The rosettes are 15 µm in diameter.

In the mesocarp of the fruit wall are seen small clusters of prismatic crystals (Figure 3.3). Starch grains are occasionally seen in the endosperm cells (Figure 3.4) they are circular and concentric.

Powder Microscopic characters

Microscopic study of powder revealed the presence of fragments of pericarp is seen in surface view. These fragments possess cells, which are polygonal in outline with very thick anticlinal walls. Some cell inclusions are also seen in the powder. Calcium oxalate crystals are located in the cells of the pericarp. Isolated parenchyma cells are abundant in the powder. The cells are irregular shape or spherical to elongated. They are thin walled and do not possess any inclusions.

Sclereids

Sclereids of different shape and size are abundant in the powder they are all lerachysclereids types. The sclereids are isodiametric, elongated, or irregular in shape; they have thick, lignified walls and narrow lumen. The walls have wide canal line pits, the sclereids are seen either isolated or in large masses.

Trichomes are non glandular, occasionally seen in the powder. They are unicellular, unbranched and thick walled. The lumen is wide. The trichome is gradually tapering towards the top, it is 700 µm long and 400 µm thick at the base. These quantitative data are useful for setting standards for crude drug.

Seed; Ovary are 2–5 celled ovules 2 in each cell. Styles subulate are entire. Drupe with a single bony tuberculate stone divided into 1–5 seeded cells. Seeds are pendulous, testa hard albumen fleshy cotyledons broad.[32]

DISCUSSION

In the view of developing a customary monograph for this species, the scanning electron microscope [SEM] studies of leaves of *Elaeocarpus oblongus* had been evaluated. The results show variation in the cuticular ornamentation compared to stomata and trichomes of *E.oblongus* with other species. These distinguished characters found to be useful in identifying the members of this species. The cuticular features were aiding in deciding the taxonomic positions of the number of Elaeocarpaceae family.[33]

A chemical character of the *Elaeocarpus* species almost habitually contains either myricetin or gallic acid. Four out of seven species have been found to contain mearnesetin, rare methyl ether of myricetin. The leaf of the *Elaeocarpus oblongus* contains mearnsetin (flavonoid) 00.015% and myricetin, 00.19% flavonoid.[2] The qualitative analysis report showed (Table 3) the presence of primary metabolites such as Carbohydrate in the form of glucose and fructose in the fruit. Phenolic compounds, tannin, flavonoids, saponins and sterols and fatty acids were the secondary metabolites in the fruit.

Leaf, stem and stem bark of *Elaeocarpus oblongus* extracts was evaluated biologically for various activities such as antibacterial, antiviral, antifungal and effect on respiration and effect on cardiovascular and effect on isolated ileum and effect on isolated rat uterus and effect on CNS and cross behavior and effect of hypothermia analgesia and supra maximal electroshock seizure pattern test and diuretic and anti inflammatory. It was reported that the LD_{50} of the leaf, stem and stem bark was 464 mg/kg, 1000 mg/kg and 175 mg/kg respectively.[34] Stem and stem bark was not showing any potential biological activity, only leaf exhibited action on respiration. The current study has selected the much used edible fruit of this species for setting the quality control parameters. The fruits of *E. oblongus* exhibited potent antioxidant activity towards 1, 1-diphenyl-2- picrylhydrazyl free radical (DPPH·) (EC50 = 0.200 ± 0.76 and 0.250 ± 1.76 mg mL^{-1}), superoxide anion ($O2^{\cdot-}$) (EC50 = 0.50 ± 0.82 and 0. 50 ± 1.20 mg mL^{-1}), hydroxylion (˙OH) (EC50 = 0.250 ± 0.22 and 0.500 ± 0.48 mg mL^{-1}) and nitric oxide (NO) (EC50 = 0.250 ± 0.22 and 0. 250 ± 0.552 mg mL^{-1}).[35]

This fruit is susceptible to fungal growth since the fruit contains much moisture content was estimated about 12% w/w. In our antifungal studies, we had got a negative result, 10mg of the alcoholic extract of fruit in DMSO was failed to prevent the growth of *Candida albicans* in potato dextrose agar medium. However Mycorrhizal type and Dark septate endophyte (DSE) fungal association was not found in the root of *Elaeocarpus oblongus*.[36] Microscopic study of powder revealed the presence of rosette type of calcium oxalate crystals, lerachysclereids and squarish compact parenchyma cells of ground tissue are important anatomical characters and this observation were found to be identical features.

CONCLUSION

The physicochemical characters and anatomical characters, powder microscopy, and its quantitative data have been provided in this work can be used to develop the fundamental module of the contemporary monograph and also helped to identify and differentiate the closely allied species of the genus *Elaeocarpus*. The present study continuously progress to analyse the nutritive value and to isolate the important flavanoids in the fruit and screening the antinociceptive activity to prove folkloric report.

ACKNOWLEDGEMENT

We thank Mr.Rajendran and Mr.Manikandan to provide facilities to visited Edakatu to conducted survey and collected the plant specimen and fruits from the tree of *Elaeocarpus oblongus*.

CONFLICTS OF INTEREST

All authors, none to declare

ABBREVIATIONS

OEP – Outer epidermis, OGPA – Outer ground parenchyma, Ta – Tannin, VS – Vascular strand, IGPA – inner ground parenchyma, In – Integument, END – Endosperm, EM – Embryo, EP – Epidermis, GP – Ground parenchyma, SCL – sclereids, CT – cotyledon, DB – Dark stained bodies, IEP – Inner epidermis, DR – druses, OM – Organic matter, SG – starch grain, AW – Anticlinal wall, CR – crystal, EC – Epidermal cell, PA – parenchyma, NTR – Non glandular trichome, IUCN – International Union for Conservation of Nature, WHO – World Health Organization, QCMMP – Quality control methods for medicinal plant materials, PARC – Plant anatomy research centre, Zn – Zinc, Mn – Magnesium, Cu – Copper, Cr – Chromium, Pd – Lead, UV – Ultraviolet, RLS – Radial longitudinal section, TLS – Transverse longitudinal section, FAA – Formalin Acetic acid Alcohol, TBA – tertiary–Butyl alcohol, UV – Ultraviolet, EC50 – Effective

concentration, LD_{50} – Lethal Dose, CNS – Central nervous system, TLC – Thin layer chromatography, FAA – Formalin Acetic acid Alcohol, DMSO – Dimethyl sulphoxide, DSE – Dark septate endophyte, (DPPH·) – 1, 1-diphenyl-2- picrylhydrazyl free radical, ($O2^{\cdot-}$) – Superoxide anion, (·OH) – Hydroxyl ion, (NO) – Nitric oxide, nm – nanometer, mm – milimeter.

REFERENCES

1. Coode MJE. "Elaeocarpus in New Guinea - new taxa in the Debruynii subgroup of the Monocera group, Contributions to the Flora of Mt Jaya, V". Kew Bulletin, Kew: United Kingdom; 2001.
2. Lalchand S, Dasgupta S, Chattopadhyay SK, Ray AB. Chemical investigation of some Elaeocarpus species. Planta medica.1977; 32:197–9.
3. Gagan Shah, Prabh Simran Singh, Mann AS, Shri R. Scientific basis for the chemical constituent and therapeutic use of Elaeocarpus species: A Review. Int J of Insti Pharm and Life Sci. 2011; Jul-Aug 11:267–78.
4. Amid Dadhich, Anirudha Rishi, Gargi Sharma, Subhash Chandra. Phytochemicals of Elaeocarpus with their therapeutic value A Review. Int J Pharm Bio Sci. 2013 Jul; 43:591–8.
5. Nair NC, Henry AN. Flora of Tamilnadu. Howrah, India :Botanical survey of India; 1983.
6. Chopra RN, Nayer SL, Chopra IC. Glossary of Indian Medicinal Plants, 3rd ed, New Delhi: Council of Scientific and Industrial Research; 1992.
7. Council of Scientific and Industrial Research (CSIR), The wealth of India: a dictionary of Indian raw materials and industrial products, Raw materials, revised edition, Publication and information directorate, New Delhi, India; 1992.
8. Pullaiah T. Encyclopedia of world medicinal plants. New Delhi: Regency Publication; 2006.
9. Jain SK. Dictionary of Indian folk medicine and ethnobotany. New Delhi: Deep publication; 1991.
10. http://municipality.tn.gov.in/ooty/abt-muni.htm
11. Statistical hand book-The Nilgiris. nilgiris.nic.in/images/districthandbook0809.pdf
12. PK Manigandan PK, Activity concentration of radionuclides in plants in the environment of Western Ghats. Iran. J. Radiat. Res., 2009; 72:85–90.
13. Sass JE. Elements of botanical micro technique. New York: Mc Graw Hill; 1940.
14. Johansen DA. Plant Micro technique. New York, USA: Mc Graw-Hill; 1940.
15. John ES. Elements of botanical micro technique. 1st ed. Mc Graw hill; NewYork: 1940.
16. Kraus JE, Arduim M. Manual basico de metodos em morfologia vegetal. Editora da universidade rural, Seropedica; 1997.
17. O'Brien TP, Feder N, Mc Cull ME. Polychromatic Staining of Plant Cell walls by toluidine blue. O.Protoplasma. 1964; 59:364–73.
18. Brain KR, Turner TD. The Practical evaluation of Phytopharmaceuticals. Bristol: Wright Scientechnica; 1975.
19. Kokate CK. Practical Pharmacognosy. 4th ed. New Delhi, India; Vallabh Prakashan; 2005.
20. Kay LA. The Microscopical Studies of Drugs. Ist ed. London: Bailliere & Cox; 1938.
21. Trease GE, Trease NC. Pharmacognosy. London: Crowell-Collier & Macmillan Publishers; 1972.
22. Wallis TE. Text Book of Pharmacognosy. 5th ed. London: J & A Churchill; 1967.
23. Easu K. Plant Anatomy John Wiley & sons. New York: John Wiley & sons; 1964 and Anatomy of seed Plants. New York: John Wiley & sons; 1979
24. Chase CR, Pratt RJ. Fluorescence of powdered vegetable drugs with particular reference to development of a system of identification. J Am Pharm Assoc. 1949; 38:324–31.
25. Kokoski CJ, Kokoski RJ, Salma FJ. Fluorescence of powdered vegetable drugs under ultraviolet radiation. J Am Pharm Assoc.1958; 47:715–7.
26. Vogel AIC. A Text Book of Macro and Semi-Micro Qualitative inorganic analysis. London: Longman, Green- Co. Ltd; 1953.
27. World health organization. Quality Control Methods for Medicinal plants. Geneva; 1998.
28. Ayurvedic Pharmacopeia of India. Ministry of Health and Family Welfare, Department of Ayush, New Delhi; 1999.
29. Wagner H, Bladt S. Plant Drug Analysis In: A Thin Layer Chromatography Atlas. 2nd ed. Springer-Verlag: New York; 1996.
30. Stahl E. Thin layer chromatography. A Laboratory Handbook. 2nd ed. New York: Springer-Verlag Berlin; 1969.
31. Deb AC. Fundamentals of Biochemistry.7th ed. Kolkata: New Central Book Agency; 1998.
32. Gamble JS, Fischer CEC. Flora of Presidency of Madras. London: Adlard and Sons Ltd; 1924.
33. Singh HP, Dube VP. Scanning Electron Microscope studies of the leaves in the family Elaeocarpaceae. Phytomorphology. 1991; 41: 257–65.
34. Abraham Z, Bhakuni DS, Garg HS, Geel AK, Mehrotra BN, Patnaik GK, et al. Screening of Indian plants for biological activity. Ind J of Exp Bio.1986; 24:48–68.
35. Ramakrishnan S, Jagathala M, Engoor SA, Paul Hephzibah C, Mangalanandan SL et al, Antioxidant Capacity of *Rubus niveus* Thunb. and *Elaeocarpus oblongus* Gartn. Fruits: 2011. http://www.globalsciencebooks.info/JournalsSup/images/WebJournals/FOOD_5(SI1)IssueInformation.pdf.
36. Govindaraj, Bagyalakshmi, Thangavelu Muthukumar, Kullaiyan Sathiyadash, Vellaisamy Muniappan. Mycorrhizal and dark septate fungal associations in shoal species of Western Ghats, southern India. *Mycoscience*. 2010; Jan 511:44–52.

Screening for Antidepressant-Like Effect of Methanolic Seed Extract of *Avena Sativa* using Animal Models

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ABSTRACT

Depression affects about up to 20% of the population across the globe.The present study was designed to screen antidepressant activity of methanolic seed extract of *Avena sativa* (MSEAS). An *in-vivo* experimental methods were designed such as behavioral models like Forced swim test (FST), Tail suspension test (TST) and based on mechanism of action i.e., Antagonism of Apomorphine induced hypothermia on Swiss male albino mice. MSEAS 100 and 200 mg/kg, p.o were administered daily for 7 days. Fluoxetine 25mg/kg p.o was standard antidepressant drug in behavioral models and Desipramine 20mg/kg p.o in Apomorphine induced hypothermia.The methanolic extract produces a significant antidepressant effect in both FST and TST as they reduce the immobility. It was also found, effective in antagonizing or reversing hypothermia produced by apomorphine. The Anti-depressant activity of methanolic seed extract of *Avena sativa* was found to be significant at low doses (100mg/kg, po). The present study clearly demonstrated that *Avena sativa* exerts an antidepressant effect in these two behavioral models. The flavonoid components of MSEAS might be interacting with adrenergic system in mediating the anti depressant effect of *Avena sativa*.

Keywords: *Avena sativa,* forced swim test, tail suspension test, Apomorphine induced hypothermia

INTRODUCTION

Depression is a mental disorder characterized by a pessimistic sense of inadequacy and a despondent lack of activity with sad feelings of gloom, inadequacy and is present with depressive mood, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, low energy, and poor concentration.[1] It is mainly caused by decreased brain levels of mono-amines like nor adrenaline, dopamine and serotonin.It is about twice as common in women as in men.[2]

Although a number of synthetic drugs are being used as standard treatment for clinically depressed patients, they have adverse effects that can compromise the therapeutic treatment. Thus, it is worthwhile to look for antidepressants from plants with proven advantage and favorable benefit-to-risk ratio. A number of medicinal plants and medicines derived from these plants have shown antidepressant properties by virtue of their medicinal constituents.

Avena sativa Linn.commonly known as oats belongs to the family of Gramineae; Poaceae.[3] The primary chemical constituents includes saponins (Avenacosides A and B), flavonoids, starch, (trigonelline, avenine, gramine), steroids, calcium, B-vitamins, lysine, methionine and alkaloids such as gramine, they also contain iron, manganese, zinc.[4–6] The plant used for depression, exhaustion, stress reduction, nervous system tonic, sexual performance, detoxification, nicotine cravings, fibroids, candidiasis, attention deficit disorder, respiratory and immune support and migraine headaches.[7]

There is no significant work has been carried out on the antidepressant effect of this plant extract till date. Hence, the present study was designed to screen the antidepressant activity of *Avena sativa* using different animal models in mice.

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DOI: 10.5530/pj.2014.3.13

MATERIALS AND METHODS

Materials

The plant material of *Avena sativa* seeds used for the investigation was collected from a local distributor in Tirupathi in the month of February 2012. The plant was identified and authenticated by Dr. K MadhavaChetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, Chittoor district, Andhra Pradesh. Apomorphine (Sigma life sciences, Bangalore), Fluoxetine (Fludac, Cadila), Desipramine (Sigma life sciences, Bangalore) and UgoBasile Rectal Thermometer. All other solvents and chemicals used were of analytical grade.

Preparation of extract

About 1kg of seeds of *Avena sativa* were shade dried at room temperature and grinded coarsely (40 mesh size) before extraction. The seeds were extracted by Soxhlet apparatus by using solvent methanol. The resulting extract was concentrated in vacuum under reduced pressure using rotary evaporator and dried in desiccator and the percentage yield was found to be 42%. Further, extract was subjected to preliminary phytochemical analysis.[8]

Animals

The albino mice (20-30g) of either sex were used throughout the experimentation. After randomization into various groups, animals were acclimatized for a period of 10 days under standard husbandry conditions i.e. room temperature of 27±3^{0}C, relative humidity of 65±10%, 12 h light/dark cycle. All the animals were fed with standard rodent pellet diet supplied by M/s. Rayans biotechnologies Pvt. Ltd., Hyderabad and water *ad libitum*. Ethical clearance for performing experiments on animals was obtained from Institutional Animal Ethic Committee (Reg. No. 1236/c/08/CPCSEA)

Acute oral toxicity study

Determination of Maximum Tolerance Dose (MTD)

Acute toxicity studies were performed for selected plant methanolic extracts according to the toxic classic method as per guidelines 423 prescribed by OECD,[9] 2001 using female albino mice. The extracts showed neither visible sign of toxicity nor mortality.

Functional Observational Battery (FOB)

The Functional Observational Battery is a non-invasive procedure designed to detect gross functional deficits resulting from exposure to chemicals and to better quantify neurotoxic effects. A group of 6 mice of either sex were used to study the effect on general behavioral pattern. The mice were fasted for 3hrs prior to the oral administration of compound. Observations were taken at ½, 1, 2 and 4hrs intervals. The various parameters, their corresponding scores as per the method of Irwin[10–13] were recorded.

Experimental protocol

Mice were randomly divided into four groups and each group having six animals. Group I received distilled water 10ml/kg, p.o and served as a control; group II received standard antidepressant drug Fluoxetine(25mg/kg)/ Desipramine(20 mg/kg) for 7&14 successive days ; group III and IV received methanolic seed extract of *Avena sativa* (MSEAS) 100 and 200 mg/kg, p.o respectively.

SCREENING FOR ANTIDEPRESSANT ACTIVITY

Behavioral Tests

Forced Swim Test (FST)

The FST is the most widely used *in vivo* screening model for assessing antidepressant activity. Experiment was carried out in narrow glass cylinder (13 cm in diameter × 24 cm high) containing water (25°C) to a depth of 10 cm, from which they cannot escape. All the animals were fasted for 3hrs prior to the oral administration of vehicle/standard/test drugs. Thirty minutes later, the animals were subjected to swim for 6 minutes; the first two minutes the animal is allowed to adjust to the new conditions; the next four minutes the immobility time was measured with a stopwatch at 30, 60,120 and 240 minutes. Immobility time was the time during which the animals will be necessary to keep afloat.[14–17] Here the standard drug is Fluoxetine 25mg/kg p.o.3

Tail Suspension Test (TST)

The control, test and standard drugs were administered p.o., 60 minutes prior to testing. The mice were suspended on the edge of a shelf 58cm above the table top by adhesive tape placed approx. 1cm from the tip of tail. The duration of immobility was recorded for the period of 6 minutes by using stopwatch. After the initial period

of vigorous motor activity, the mice became still. Mice were considered immobile when they hanged passively and completely motionless. The duration of immobility time was recorded before the treatment and 60 minutes after the treatment.[18–19] Here the standard drug is Fluoxetine 25mg/kg p.o.

Based on Mechanism of Action

Apomorphine induced hypothermia

All the animals were fasted for 3 hrs prior to oral administration of vehicle/standard/test drugs. One hour after oral administration of the test compounds or the vehicle, 16mg/kg apomorphine was injected s.c. to the animals. The rectal temperature of each mouse was measured by an electronic thermometer at 10, 20, 30, 60 and 120 minutes after apomorphine treatment and the degree of hypothermia was determined. During the entire experiment, animals were housed in groups in glass jars at room temperature.[20-21] Here the Standard drug is Desipramine 20mg/kg p.o.

Statistical analysis

Results will be presented as mean±SEM. The data will be subjected for statistical analysis by One way analysis of variance (ANOVA) followed by Dunnet's t test and P<0.05*, 0.01** and 0.001*** were considered as significant.

RESULTS

Phytochemical screening

The Percentage yield of extract was found to be 42.23% w/w and the preliminaryphytochemical analysis showed the presence of carbohydrates, alkaloids, flavonoids, steroids, glycosides, saponins, amino acids, gums and mucilage.

ACUTE ORAL TOXICITY STUDY

Determination of Maximum Tolerance Dose (MTD)

The results clearly indicated non-toxicity of the extracts at a dose of 2000 mg/kg. From this 20th and 10th parts were selected as dose for the experimental study. Hence LD_{50} determination was not possible and all the extracts tested are considered safe and nontoxic according to OECD guidelines 423.

Functional Observational Battery (FOB)

The methanolic seed extract of *Avena sativa* was subjected to FOB, which is a non-invasive procedure to detect gross functional deficits basing upon various behavioral parameters. The scoring of various parameters was shown in (Table1). The results showed an increase in spontaneous motor activity, ataxia and stereotypic behaviors like rearing and grooming.

SCREENING FOR ANTIDEPRESSANT ACTIVITY

Forced Swim Test (FST)

The result of the effect of methanolic seed extract of *Avena sativa* on the duration of immobility was shown in figure 1. The animals treated with 100mg/kg, p.o of MSEAS and standard antidepressant Fluoxetine 25mg/kg, p.o showed significant decrease in immobility time but not 200mg/kg, p.oof MSEAS when compared with control group.

Tail Suspension Test (TST)

The results were presented in (Figure 1), revealed that the immobility time was significantly decreased in animals treated with 100mg/kg , p.o of MSEAS and standard antidepressant Fluoxetine 25mg/kg ,p.o but not 200mg/kg ,p.o of MSEAS when compared with control group.

Apomorphine induced hypothermia

In this test, animals treated with two doses of MSEAS (100 & 200mg/kg, po) and standard antidepressant Desipramine (20mg/kg, po) showed significant antagonism of hypothermia induced by Apomorphine (16mg/kg, sc) when compared with control group and the results are showed in (Figure 2).

The percentage inhibition of immobility time in Forced Swim Test, Tail Suspension Test and temperature in Apomorphine induced hypothermia were calculated according to the following formula and results were shown in (Table 2).

% inhibition of immobility time

$$= \frac{\text{control standard/text}}{\text{control}} \times 100$$

Table 1. Effect of MSEAS in Functional Observation Battery of Control group, Test dose (100mg/kg, p.o), Test dose (200mg/kg, p.o)

Behavioral parameters	Normal score			30 minutes			60 minutes			120 minutes			240 minutes		
	C	T_1	T_2	C	T_1	T_2	C	T_1	T_2	C	T_1	T_2	C	T_1	T_2
Spontaneous Motor Activity	4	4	4	4	5	5	4	5	5	4	5	5	4	5	5
Respiration	4	4	4	4	4	4	4	4	4	4	5	5	4	5	5
Ataxia	0	0	0	0	1	1	0	1	1	0	1	1	0	1	1
Inclined plane	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Body temperature	38.1	38.1	38.1	38.35	37.6	39.2	38.51	38.1	39.6	38.37	36.9	39.2	38.21	37.6	39.4
Convulsions	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sound & Touch Response	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pinna reflex, Corneal reflex, Righting reflex	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Analgesia	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Writhing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stereotype behaviour	0	0	0	0	1	1	0	2	2	0	2	2	0	2	2
Body tone	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Limb tone	4	4	4	4	4	4	4	4	4	3	3	4	3	3	3
Urination	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diarrhoea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pupil size	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Ptosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Struab tail	0	0	0	0	2	2	0	2	2	0	2	2	0	2	2
Catalepsy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hypothermia	0	0	0	(0.25)	–0.5	–1.1	(0.41)	–1.5	–1.5	(0.27)	–1.1	–1.1	(–0.11)	–1.3	–1.3
Startle response	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyanosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exopthalmus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

C= Control group, T_1= Test dose of 100 mg/kg , T_2= Test dose of 200 mg/kg

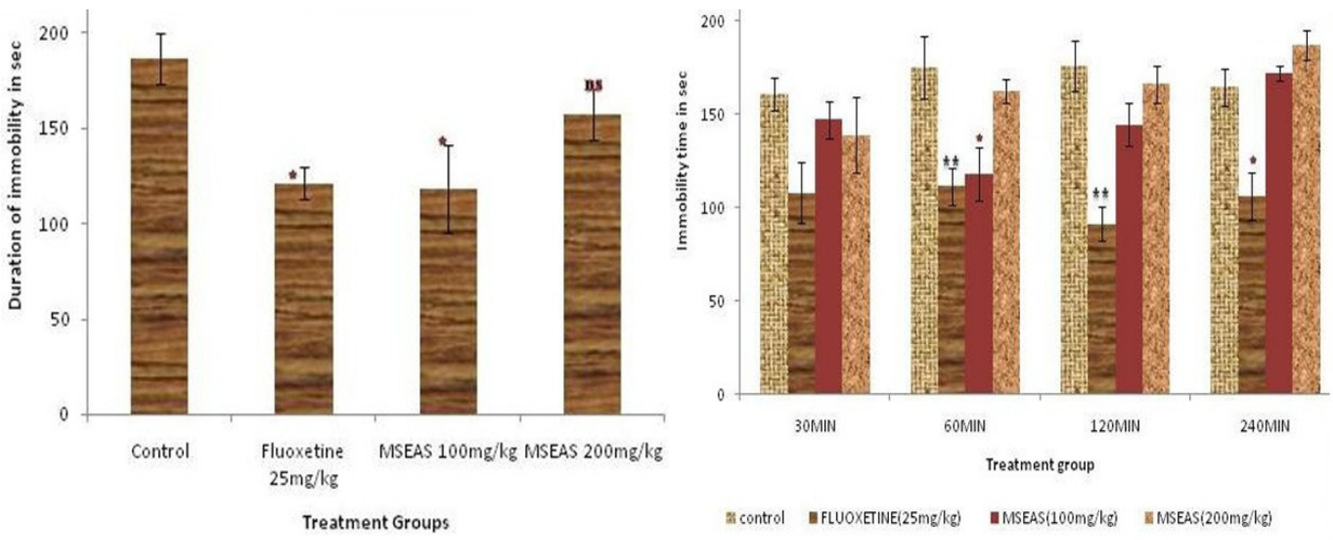

Figure 1. Histograms of Mean ± SEM of immobility time in Forced Swim Test and Tail Suspension Test.

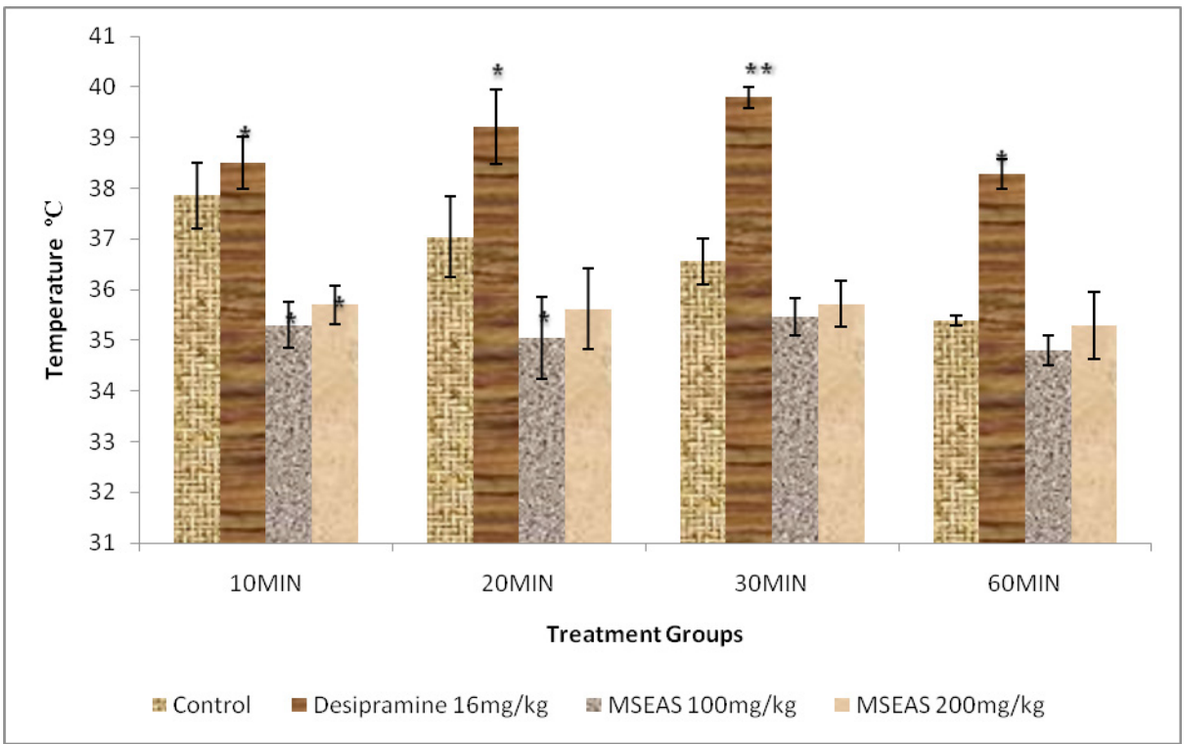

Figure 2. Histograms of Mean ± SEM of temperature in antagonism of Apomorphine induced hypothermia.

Table 2. Effect of MSEAS on % inhibition of immobility time in Forced Swim Test, Tail Suspension Test and temperature in Apomorphine induced hypothermia

Time (Min.)	% inhibition of immobility time in Forced Swim Test			% inhibition of immobility time in Tail Suspension Test			% Inhibition of temperature in Apomorphine induced hypothermia		
	TREATMENT			TREATMENT			TREATMENT		
	Fluoxetine (25mg/kg)	MSEAS (100mg/kg)	MSEAS (200mg/kg)	Fluoxetine (25mg/kg)	MSEAS (100mg/kg)	MSEAS (200mg/kg)	Desipramine (20 mg/kg)	MSEAS (100mg/kg)	MSEAS (200mg/kg)
30	33.16	8.69	13.78	–	–	–	8.92	3.66	2.24
60	36.52	32.87	7.30	34.87	36.48	15.45	7.65	2.02	0.50
120	48.2	17.95	5.56	–	–	–	8.00	5.92	8.62
140	35.52	4.62	13.74	–	–	–	–	–	–

MSEAS: methanolic seed extract of *Avena sativa*

DISCUSSION

Since the introduction of the herbal medicines, many people were impelled to consider the importance of many herbs for treating several forms of disorders.[22] Modern day lifestyle leads to numerous stress conditions, among which depression is a widely prevalent senile neurological disorder. It is mainly caused by decreased brain levels of monoamines like Noradrenaline, Dopamine and Serotonin. Depression is a mental disorder associated with lot of morbidity due to its high incidence in the community. Hence it is necessary to look for Anti depressants with proven advantage and favorable benefit-to-risk ratio. Although a number of synthetic drugs are being used as standard treatment for clinically depressed patients, they have adverse effects that can compromise the therapeutic treatment. Therefore, there is an immense requirement for alternative remedies for depression.The present work was subjected to investigation for the evaluation of the Anti depressantactivity of methanolic seed extract of *Avena sativa* in animal models.

In Acute Oral Toxicity study, MSEAS did not show any lethal effect even up to the doses of 2000mg/kg, po and complete absorption of drug through GIT was observed

and thus the test doses of 100 & 200mg/kg, po were used. The Functional Observational Battery is a non-invasive procedure designed to quantify neurotoxic effects. It is used for assessing the behavioral parameters in the mice when exposed to chemicals. The behavioral parameters observed in MSEAS extract were increase in spontaneous motor activity; ataxia and stereotypic behavior like rearing and grooming were observed.

For the purpose of investigation of antidepressant activity, two animal models viz.,the forced swim test and tail suspension test were used. These tests were quite sensitive and relatively specific to all major classes of Anti depressants. The immobility displayed by rodents when subjected to unavoidable stress such as FST & TST are thought to reflect a state of despair or lowered mood, which are thought to reflect depressive disorders. In addition, immobility time has been shown to be reduced by treatment with antidepressant drugs.

Results showed that the administration of the MSEAS produced a diminution of duration of immobility time of mice exposed to the both FST & TST. In the present study, the MSEAS (100mg/kg, po) administered to mice produced significant antidepressant effect in both FST & TST models and their efficacies were found to be comparable to standard drug Fluoxetine (25mg/kg, po).

For the assessment of mechanism of action of MSEAS, antagonism of Apomorphine induced hypothermia model was used to know MSEAS acting through Nor-adrenaline.Antagonism against Apomorphine induced hypothermia can be regarded as a hint for antidepressant activity through Nor-adrenaline uptake. Compounds with a marked Nor-adrenaline or Dopaminergic components are active against Apomorphine induced Hypothermia but not through seratonergic system. In this model, two doses of MSEAS (100 & 200mg/kg, po) and Desipramine (20mg/kg, po) significantly antagonized the Apomorphine induced hypothermia (16mg/kg, sc) when compared with control, representing that MSEAS may be acting through Adrenergic system but not through seratonergicsystem.

From all the above, the antidepressant activity of methanolic seed extract of *Avena sativa* was found to be significant at low doses (100mg/kg, po). The flavanoid components of MSEAS might be interacting with adrenergic system in mediating the anti depressant effect of *Avena sativa.*

CONCLUSION

The MSEAS contained carbohydrates, alkaloids, flavonoids, steroids, glycosides, saponins, amino acids, gums and mucilage. It has not produced any lethal effect even up to the dose level of 2000mg/kg, p.o during acute oral toxicity study. The findings of the present investigation suggests that the Anti-depressant activity of MSEAS was significant at lower dose of 100mg/kg , p.o in Forced swim test, Tail suspension test and also Antagonism of Apomorphine induced Hypothermia indicated that MSEAS is showing the Antidepressant activity by acting through Adrenergic system.

However, more extensive pharmacological studies of this plant are required for complete understanding of the Antidepressant activity of methanolic seed extract of *Avena sativa.*

REFERENCES

1. "Depression". The National Institute of Mental Health (NIMH). Retrieved from NIMH, component of the U.S. department of health and human services.
2. Andrade L, Caraveo-Anduaga JJ, Berglund P *et al*. The epidemiology of major depressive episodes: Results from the International Consortium of Psychiatric Epidemiology (ICPE) Surveys. Int J Methods Psychiatr Res., 2003; 121: 3–21.
3. C.P. Khare, Indian Medicinal Plants, Springer publication, 2007, p 73–74.
4. Pecio L, Wawrzyniak-Szołkowska A, Oleszek W, Stochmal A. Rapid analysis of avenacosides in grain and husks of oats by UPLC-TQ-MS, Food Chem., 2013;1413: 2300–04.
5. Wei-Ku Zhang, Jie-Kun xu, Li Zhang, Guan-Hua DU. Flavonoids from the bran of *Avena sativa*.ChinJ Nat Med.,2012;102: 110–14.
6. Janatuinen EK, Kemppainen TA, Julkunen RJK, Kosma VM, Maki M, Heikkinen M and Uusitupa MI. No harm from five year ingestion of oats in celiac disease, Gut, 2002; 50: 332–35.
7. Singh R, De S, Belkheir A. *Avena sativa* (Oat), a potential neutraceutical and therapeutic agent: an overview.Crit Rev Food SciNutr., 2013; 532: 126–44.
8. World Health Organization Expert Committee, Quality Control Methods for Medicinal Plant Materials, WHO, Geneva. 1998; 9: 22–34.
9. OECD, Organization for Economic Co-operation and Development Guidelines for the Testing of Chemicals, Test no. 423: Acute Oral Toxicity-Acute Toxic Class Method; 2001.
10. Irwin S. Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia 1968; 13: 222–57.
11. Rogers DC, Peters J, Martin JE, Ball S, Nicholson SJ, Witherden AS, Hafezparast M, Latcham J, Robinson TL, Quilter CA, Fisher EM. SHIRP A. a protocol for behavioral assessment: validation for longitudinal study of neurological dysfunction in mice. NeurosciLett., 2001; 306: 89–92.
12. Virginia C. Moser. Functional Assays for Neurotoxicity Testing. ToxicolPathol., 2011; 39: 36–45.

13. Boucard A, Betat AM, Forster R, A Simonnard G Froget. Evaluation of neurotoxicity potential in rats: The functional observational battery. CurrProtocPharmacol., 2010; 51: 10.12.1–10.12.9.
14. Ozturk Y, Aydin S, Tecik B, Husanu Can Baser K. Effect of essential oils from certain Ziziphora species on swimming performance in mice. Phytother. Res., 1995; 9: 222–27.
15. Porsolt R, Anton G, Jafre M. Behavioural despair in rats: A new model sensitive to antidepressant treatments. Eur. J. Pharmacol., 1978; 47: 379–91.
16. Maity TK, Mandal SC, Saha BP, Pal M. Effect of *Ocimum sanctum* roots extract on swimming performance in mice. Phytother. Res., 2000; 14: 120–21.
17. Porsolt RD, Bertin A, Jalfre M. Behaviour despair models in mice: a primary screening test for antidepressants, Arch. Int. Pharmacodyn., 1977; 229: 327–36.
18. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. Psychopharmacology 1985; 367–70.
19. Vangeois JM, Passera G, Zuccaro F, Costentin J. Individual differences in response toimipramine in the tail mouse suspension test, Psychopharmacology, 1997; 134: 387–91.
20. Porsolt RD. Animal models of depression: utility for transgenic research. Rev. Neurosci., 2000; 111: 53–58.
21. Willner P. The validity of animal models of depression. Psychopharmacology,1984; 831:1–16.
22. Ganapaty S, Ramaiah M, Ramakrishna P, Reddy DN. Scientific validation and formulation of three Indian Folklore medicinal plants, Journal Pharm Res., 2013; 6: 823–35.

Antianaphylactic, mast cell stabilizing and antiasthmatic activity of AHR-1 (a polyherbal formulation)

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ABSTRACT

Objective: This work was mainly aimed to study the anti-anaphylactic, mast cell stabilizing and antiasthmatic activity of AHR-1 (a polyherbal formulation) which contain various herbal extracts. **Methods:** The antianaphylactic activity of AHR-1 was evaluated in rats using active anaphylaxis model.Rats were then observed for onset of symptoms of anaphylaxis reaction such as increased respiratory rate, dyspnea, cyanosis and mortality. Serum IgE, leukocyte, eosinophil countand % polymorphs were calculated. Mast cell stabilizing effect was investigated by in vitro challenge of antigensensitized rat intestinal mesenteries. Antiasthmatic effect was studied in guinea pigs using histamine-induced bronchospasm, in which occurrence of preconvulsive dyspnea (PCD) was noted as end point. **Results:** Anaphylactic shock caused by intravenous antigen challenge showed 83% mortality with a significant ($P<0.001$) increase respiratory symptom score.Treatment with AHR-1(250 and 500 mg/kg) reduced the mortality and respiratory symptom score ($P<0.05$,$P<0.001$), respectively. AHR- 1 (250 and 500 mg/kg) significantly and dose dependently decreased Serum IgE ($P<0.05$, $P<0.001$), AEC ($P<0.05$, $P<0.001$), total leukocytes ($P<0.05$, $P<0.01$) and % polymorphs ($P<0.01$,$P<0.001$), respectively as compared to sensitized control group. Sensitized control rats were produced a significant (79%) mesenteric mast cell degranulation, but pre-treatment with AHR-1 (100 and 200μg/ml) produced in a significant ($p<0.001$) reduction in the number of degranulated mast cells when challenged with horse serum. AHR-1 significantly increased the time of PCD ($P<0.001$) as compared to control. **Conclusion:** From these finding, we concluded that AHR-1 is might be effective in treatment various hypersensitivity reactions like anaphylactic shock and asthma.

Keywords: Horse serum, AHR-1, IgE, Respiratory score.

INTRODUCTION

Allergy is one of the common diseases that affect mankind with diverse manifestations. The prevalence of allergy and asthma has risen in recent years despite the general health improvement in the population.[1] Asthma affects about 300 million people worldwide and it has been estimated that a further 100 million will be affected by 2025.[2] It is known that asthma can be triggered by various factors: allergens, drugs, respiratory infection, dust, cold air, exercise, occupational stimuli, chemicals, histamine, etc.[3] Anaphylaxis is mediated by histamine released in responses to cross-linking of IgE bound to FcϵR1on mast cells. Mast cell activation causes process of degranulation that result in releasing of mediators, such as histamine and an array of inflammatory cytokines.[4,5] In spite of the voluminous literature on the subject, the treatment of allergic diseases continues to be far from satisfactory. The disease statistics clearly necessitates the increasing need for drugs targeting the mechanisms involved in eosinophil and differential leukocytes activation and accumulations, for the management of asthma. The available treatment options for upper and lower respiratory tract allergic diseases have major limitations owing low efficacy, associated adverse events, and compliance issues.[6] Ayurveda, an ancient system of Indian medicines, has described several drugs from indigenous plant sources for the treatment of bronchial asthma and allergic conditions. AHR-1 is a polyherbal formulation containing herbal extract listed in (Table 1).

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DOI: 10.5530/pj.2014.3.14

Table 1. Composition of each hard gelatin capsule of AHR-1

Sr. No.	Common name	Latin name	Qty in mg/ caps
1	Ardusi	*Adhatoda vasica*	63
2	Siris	*Albizzia lebbeck*	25
3	Shallaki	*Boswellia serrata*	22
4	Devdar	*Cedrus deodara*	42
5	Bharangmool	*Clerodendron serratum*	25
6	Haldi	*Curcuma longa*	28
7	Pushkarmool	*Inula recemosa*	11
8	Tulsi	*Occimum sanctum*	28
9	Katuki	*Picrorrhiza kurroa*	28
10	Piper	*Piper longum*	28
11	Kantkari	*Solanum xanthocarpum*	31
12	Sharpunkha	*Tephrosia purpurea*	12
13	Guduchi	*Tinospora cordifolia*	25
14	Arkaparni	*Tylophora asthamatica*	11
15	Nagod	*Vitex negundo*	18

Adhatoda vasica is documented for its potent anti-inflammatory[7] antiallergic and antitussive activities[8], bronchodilatory and smooth muscle relaxant activity.[9] *Albizzia lebbeck* also known as tree of happiness is extensively used in various traditional medicines. In Chinese system of medicine it is used for relieving stress, anxiety and depression.Whereas in Indian system of medicine it is mainly used in allergic conditions such as allergic asthma, urticaria etc. It was reported that *Albizzia lebbeck* having anti-histaminic and mast cell stabilizing property[10], anti-inflammatoty.[11] *Curcuma longa* (turmeric) contains curcumin, demethoxycurcumin and bisdemethoxycurcumin. The traditional uses of turmeric or natural curcuminoids in folk medicine are multiple, and some are based on their antioxidant, anti-inflammatory and antiallergic properties which have been confirmed by experimental study.[12] *Ocimum sanctum* has been reported to protect against histamine as well as pollen induced bronchospasm in guinea pigs and inhibited antigen induced histamine release from sensitized mast cells.[13] *Solanum xanthocarpum* having strong bronchodilator effect along with anti-inflammatory activity.[14,15] *Clerodendron serratum* traditionally used for treatment of asthma, bronchitis, inflammation.[16] *Piper longum* has been shown to reduce the anaphylaxis in rats and protect guinea pigs against antigen induced bronchospasm.[17] *Vitex negundo* has been reported to possess mast cell stabilizing activity.[18] It was also reported that *Tephrosia purpurea* having mast cell stabilizing activity.[19] *Boswellia serrata* is known as Salai Guggal, contain boswellic acid has been reported for antiasthmatic and anti-inflammatory activity by virtue of inhibition of leukotriene biosynthesis via 5-lipoxygenase.[20,21] It was demonstrated that *Cedrus deodara* having anti-allergic and anti-asthmatic property.[22] *Inula racemosa* is an ornamental plant of the Asteraceae family that is used both internally as well as externally in ayurveda. Externally it is used as antiseptic, antifungal activity and internally it is used for cough, bronchial asthma, dyspepsia, and amenorrhea as well as dysmenorrheal.[23] It was shown that *Picrorhiza kurroa* had properties of preventing allergen and PAF induced bronchial obstruction in guinea pigs.[24] *Tinospora cordifolia* has been shown to produce anti-allergic and bronchodilator activity.[25] It was shown that *Tylophora asthmatica* had anti-asthmatic activity.[26]

There is lack of scientific data regarding the effect of AHR-1 (PHF) on asthma. Therefore, the aim of study was designed to investigate the effect of AHR-1, a polyherbal formulation on the active anaphylaxis, mast cell stabilizing in rats and histamine induced bronchospasm in guinea pigs.

MATERIALS AND METHODS

Drugs and chemicals

Histamine and horse serum were procured from sigma chemicals. Prednisolone, ketotifen and toludine blue were procured from commercial source, Vadodara. Diphtheria tetanus and pertussis vaccine obtained from Serum institute of India ltd., Pune, India. All other chemicals used were of analytical grade.

Experimental animals

Wistar rats (200-250g) and guinea pigs (400-600g) of either sex, housed in standard conditions of temperature (22 ± 2°C), relative humidity 60 ± 5% and light (12 h light/ dark cycles) were used. They were fed with standard pellet diet and water *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee as per guidance of the committee for the purpose of control and supervision of experiments on animals (CPCSEA).

Acute toxicity study

The procedure was followed as per Organization for Economic Cooperation and Development -425 (OECD-425) guideline. The acute toxicity study of ethanolic extract of AHR-1 was performed in Wistar rats of either sex. In this study, increasing doses of AHR-1 (100, 200, 500,

1000, 2500 mg/kg body weight) was orally administered to groups of 3 animals for each dose after a 12 h fast. The signs and symptoms associated with the AHR-1 administration was observed after 0, 30, 60, 120, 180 and 240 min and then once a day for the next 14 days.[27] We observed that there was no any sign of toxicity or mortality up to a dose of 2500 mg/kg.

Horse serum induced active anaphylaxis model of rats

Thirty Wistar rats were sensitized by subcutaneous injection of 0.5 ml of horse serum followed by intraperitoneal injection of 0.5 ml Triple Antigen Vaccine containing 20000 million *Bordetella pertussis* organisms (Serum institute of India ltd., Pune, India). The sensitized animals were divided into 5 groups of 6 animals each. Group I served as a normal control and received distilled water, Group II served as sensitized control which received 0.5 % Sodium CMC (vehicle), groups III was treated with reference drug (Prednisolone 10 mg/kg, p.o.) and groups IV & V were administered AHR-1 at 250 and 500 mg/kg, respectively, orally, once a day for 14days. On day 14, after 2 hr of treatment, all the animals were challenged with intravenous injection (tail vein) of 0.25 ml horse serum in normal saline except Group I. They were then observed for onset of symptoms such as increased respiratory rate, dyspnea, cyanosis and mortality for 1 hr by a blind observer. The severity of respiratory symptoms was scored: 0- No visual symptoms; 2- Increased rate of respiration; 4- Increased rate of respiration with immobility; 6- Dyspnea for 10 mins; 8- Cyanosis for 10 mins; 10- Dyspnea & cyanosis for 10 mins; 12- Death.[28] Blood sample was collected from the retro orbital plexus of rats under light ether anesthesia, using glass capillaries and stored in with or without disodium ethylene diamine tetra-acetate for biochemical parameter estimation. For serum separation, test tube (without EDTA) allowing to clot in open for 15 minutes, it was centrifuged at 5000 rpm for 20 minutes for separation of serum. Serum was stored at -20°C until further estimation. Serum IgE was quantified with an ELISA kit according to the manufacturer's instruction.Total leukocyte, eosinophil count, % polymorphs were calculated.[29]

Degranulation of rat mesenteric mast cells

Adult male albino Wistar rats were sacrificed and pieces of mesentery with connecting lobes of fat and blood vessels were rapidly dissected out, wash with distilled water, placed in Ringer Locke solution (NaCl 0.9, KCL 0.42,$CaCl_2$ 0.024, $NaHCO_3$ 0.15 and Glucose1 g/l of distilled eater) and then subjected to the following treatment schedules.

- Petri dish no.1: Ringer Locke solution (sensitized control)
- Petri dish no.2: 0.1 ml of Ketotifen (20 µg/ml)
- Petri dish no.3: 0.1 ml of AHR-1 (100 µg/ml)
- Petri dish no.4: 0.1 ml of AHR-1 (200 µg/ml)

Each petridish was incubated for 15 min at 37°C. The preparation was challenged with 5% v/v horse serum for 10 min and after that, all pieces were transferred to 10% formaldehyde solution containing 0.1% Toluidine blue and kept a side for 25 min. After staining and fixation of mast cells, the excess stain was washed with distilled water followed by dehydration by absolute alcohol. Finally slide were cleared with xylene and observed under the high power of light microscope. The Percentage intact and percentage degranulated mast cells were counted.[30]

Histamine-induced bronchospasm in guinea pigs

Experimental bronchospasm was induced in guinea pigs by exposing them to histamine aerosol.[30] Guinea pigs of either sex were selected and randomly divided into two groups, each containing five animals. Group I and group II were exposed to 1% w/v of histamine aerosol in histamine chamber (Inco Ltd, Ambala, India) showed progressive dyspnea. The end point, preconvulsive dyspnea (PCD) was determined from the time of aerosol exposure to the onset of dyspnea leading to the appearance of convulsion. As soon as PCD commenced, the animals were removed from the chamber and placed in fresh air. This time of PCD was taken as day 0 value. Group I and group II were treated with the AHR-1 at dose of 250 mg/kg and 500 mg/kg, orally, once a day for 5 day, respectively, after aerosol exposure on day 0. On day 5, 2 h after the administration of drug, the time for the onset of PCD was recorded as on day 0. The percentage increase in the time of PCD was calculating by this formula.[31]

$$\text{Percentage increase in the time of PCD} = (1 - T_1/T_2) \times 100$$

Where T_1 = time for PCD onset on day 0, T_2 = time for PCD onset on day 5

Lung histology

After sacrifice, the lung tissue was rapidly dissected out and washed with saline immediately and fixed in 10% buffered formaldehyde. Paraffin-embedded specimens were cut into 5 µm-thick sections and stained with hematoxylin and eosin (H&E). The sections were examined under the light microscope (Olympus BX10, Japan) for histopathological changes and photomicrographs (Olympus DP12 camera, Japan) were taken. The pathologist performing

histopathological evaluation was blinded to the treatment as assignment of different study groups. The sections will be viewed under 10X and 40X magnifications.

Statistical analysis

All the data are expressed as mean ± SEM (n = 6). Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using a computer-based fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Acute toxicity study of AHR-1

Acute toxicity study on the Wistar rats showed no mortality and morbidity up to 2500 mg/kg dose of AHR-1. $^1/_5$ and $^1/_{10}$ doses were taken forfurther study.

Effect of AHR-1 on symptom score, IgE, AEC, total leukocytes and % polymorphs on horse serum induced anaphylactic in rats

Horse serum sensitized rats when challenged with the same antigen (0.25 ml of horse serum, i.v.) after 2 weeks; induced anaphylaxis reaction i.e. increased respiratory rate, dyspnea, cyanosis and mortality. The treatment with AHR-1 (250 and 500 mg/kg, p.o.) showed a significant ($P< 0.05$, $P< 0.001$) decreased in anaphylaxis reaction, that was reflected in decreased respiratory score. Prednisolone (10 mg/kg,p.o.) showed a significant decrease a respiratory score (Fig. 1).

Serum IgE, AEC, total leukocyte count & % polymorphs were significantly ($P<0.001$) increased in sensitized control rats as compared to normal control group. AHR-1 (250 and 500 mg/kg, p.o. for 14 days) significantly and dose dependently decreased serum IgE ($P<0.05$, $P<0.001$), AEC ($P<0.05$, $P<0.001$), total leukocytes ($P<0.05$, $P<0.01$) and % polymorphs ($P<0.01$,$P<0.001$), respectively as compared to sensitized control group. Standard drug (Prednisolone 10 mg/kg, p.o. for 14 days) showed a significant ($P< 0.001$) decrease in serum IgE, AEC, total leukocytes and % polymorphs as compared to sensitized rats (Table 2).

Effects of AHR-1 on degranulation of mesenteric mast cells

Antigen challenge resulted in significant degranulation of the mesenteric mast cells. The treatment with AHR-1 (100 and 200 µg/ml) or kitotifen (20 µg/ml) showed a significant ($P<0.001$) protective effect on mast cells degranulation due to the challenge of antigen. The protective effect of AHR-1 on mast cell degranulation was dose dependant. Kitotifen (20 µg/ml) treatment considerably a better protection compared to AHR-1 (200 µg/ml) on mast cell degranulation (Table 3).

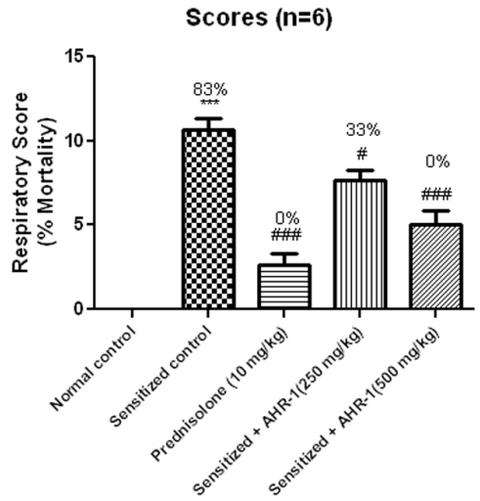

Figure 1. Effect of AHR-1 on respiratory score (% mortality) on horse serum induced anaphylactic in rats.
All the values are expressed as mean ± SEM; n = 6, $^{***}p < 0.001$ compared to normal control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to sensitized control rats

Table 2. Effects of AHR-1 on IgE, AEC, WBC, % polymorphs on horse serum induced anaphylaxis in rats

Group	Serum IgE (ng/ml)	AEC(mm3)	WBC/ cmm	%Polymorphs
I	180 ± 16	89.00 ± 7.95	4900 ± 23	50.00 ± 1.6
II	320 ± 19***	201.6 ± 9.26***	10000 ± 64***	69.00 ± 3.5***
III	180 ± 9.7###	100.4 ± 11.21###	6000 ± 27###	39.00 ± 2.7###
IV	260 ± 9.1#	158.8 ± 6.31#	8100 ± 46#	51.00 ± 3.2##
V	220 ± 13##	111 ± 9.26###	6700 ± 30##	47.00 ± 3.1###

All the values are expressed as mean ± SEM; n=6, $^{***}p < 0.001$ compared to normal control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to sensitized control rats

Effect of AHR-1 histamine-induced bronchospasm in guinea pigs

AHR-1 (250 and 500 mg/kg, p.o)significantly and dose dependently % increase in the time of PCD (P<0.001) as compared to control, following exposure to histamine aerosol on day 5 (Table 4).

Lung Histology

Normal control rats showed normal lung histology, no mural inflammation, eosinophilic inflammation is absent. (Fig. 2A). In contrast, histological section of lung of sensitized control rats showed significant changes, airway (Av) lumen is plugged by mucus and an inflammatory cells, thickened pseudo-stratified columnary epithelium (a), destruction of pulmonary artery is observed (b), accumulation of lymphocytes is constantly high (c), eosinophilic edema fluid deposition is severe (d), Parenchymal inflammation is also observed (Fig. 2B). Treatment with Prednisoloneand AHR-1 (250 and 500 mg/kg, p.o.) produce significantly less inflammatory infiltrates,alveolar lumen (Av) is normal, less thickness of epithelial and sub-epithelial layers (a), pulmonary artery is normal (b) with moderate peri alveolar inflammation, very less accumulation of lymphocytes (c) and no parenchymal eosinophilicinflammation (Fig. 2C–E).

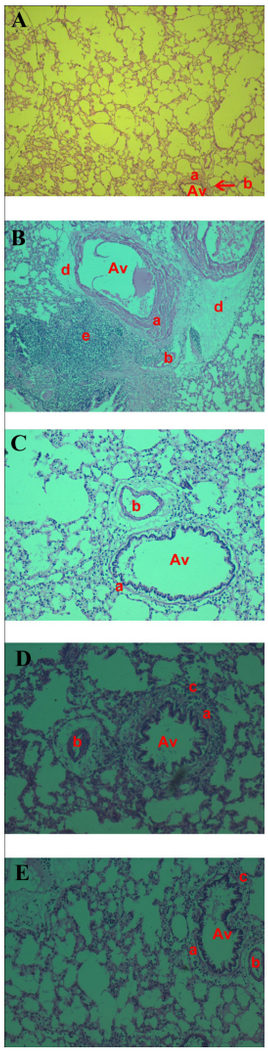

Figure 2. Histological appearance of lungs. **(A)** Normal control; **(B)** Sensitized control; **(C)** Sensitized rat treated with Prednisolone (10 mg/kg, p.o.); **(D)** Sensitized rat treated with AHR-1 (250 mg/kg, p.o.); **(E)** Sensitized rat treated with AHR-1 (500 mg/kg, p.o.).

DISCUSSION

Active anaphylaxis induced by triple antigen horse serum is a key model to study the symptomatic affect of type I allergy. The globulin fraction of horse serum acts as environmental allergen triggering the allergic responses.[32] In the present study, rats were sensitized by horse serum (0.5 ml, sc.) and then second exposure to the same antigen

Table 3. Effect of AHR-1 on mast cell degranulation

Groups	Treatment	Dose	Intact mast cells %	Degranulated mast cells%
I	Sensitized control	–	20.54 ± 1.56	79.17 ± 2.63
II	Standard(Ketotifen)	20µg/ml	79.86 ± 2.76	20.67 ± 1.47$^{###}$
III	AHR-1	100µg/ml	62.64 ± 2.65	37.67 ± 0.80$^{###}$
IV	AHR-1	200µg/m)	75.54 ± 3.54	26.33 ± 1.4$^{###}$

Values are expressed as mean ± SEM; n=6, $^{###}p$< 0.001 compared to sensitized control rats.

Table 4. Effects of AHR-1 (250 and 500 mg/kg) on histamine-induced bronchospasm in guinea pigs

Group	Preconvulsive dyspnea time(sec)		
	Before treatment (control)	After treatment	% increase in the time of PCD
I-AHR-1 (250 mg/kg)	135.8 ± 6.64	361.8 ± 18.83 ***	62.5
II-AHR-1 (500 mg/kg)	122.4 ± 6.83	391.6 ± 20.52***	68.8

Values are expressed as mean ± SEM; n=5, $^{***}p$< 0.001 when compared with control guinea pigs

(0.25ml, iv.) caused acute anaphylactic shock mediated by histamine released in responses to cross-linking of IgE bound to Fc£R1 on mast cells. Mast cell activation causes process of degranulation that resulting in the release of various mediators and cell infiltration. In our study, In sensitized control rats, intravenous antigen challenge caused anaphylactic shock in 83% of animals with mortality and significantly increased respiratory score, while in AHR-1(250 and 500 mg/kg, p.o.) treated rats, the onset of symptoms were delayed or symptoms were less severe with reduced the mortality by 33%, 0% respectively and significantly decreased respiratory score. We also found that antigen challenge resulted in significant increase in the numbers of eosinophil, lymphocytes, total leukocytes, serum IgE level. Treatment with Prednisolone and AHR-1 in antigen challenged animals significantly inhibited antigen induced hyper reactivityby decreasing the eosinophil count, % lymphocytes, infiltration of total leukocytes, serum IgE level. It was confirmed by histology of lung tissue, the section of lung tissue of sensitized animals showed airway plugging by mucus and cell, eosinophilic inflammation. Treatment with AHR-1 prevented the inflammation which led to normal lumen size and normal cellular structure.

In asthma, chronic inflammation is responsible for the bronchospasm which leads to airway narrowing and decrease in the lumen size of bronchiole.[3] It can be clearly seen in cross section of lung tissue. In the present study, treatment with AHR-1 was prevented the inflammation and bronchoconstriction as well as lead to cellular structure and normal lumen size and as compared to sanitized control rats.

It was reported that mast cell deregulation occurs when the tissues of sensitized animals brought into contact with antigen in vivo or in vitro, which is an important feature of anaphylaxis.[33] In the present study, we were finding that AHR-1 shown protection against the mast cell degranulation in antigen sensitized animals. AHR-1 (200 µg/ml) protects the degranulation of mast cell comparable to kitotifen.This indicates that AHR-1 is effective in stabilizing the mast cell. Anti-anaphylactic and mast cell stabilizing activity of AHR-1 might be due to the presence of herbal extracts, which are known for their mast cell stabilizing property against antigen-antibody reaction or due to inhibition of IgE antibody production, which is accountable for mast cell degranulation.[13]

Asthma is chronic disorder characteristic by bronchospasm and inflammation. It is triggered by exposure of allergen, irritants, cold air or exercise which releases the inflammatory mediator like histamine, acetylcholine and leukotrines that directly causes the bronchospasm.[34,35] Exposure of histamine aerosol (1% w/v) to cause the bronchospasm in the form of PCD in guinea pigs. In our study, we observed that latent period of PCD was significantly increased in AHR-1 treated animals. These results suggested AHR-1 had bronchodilator activity.

CONCLUSION

We observed that AHR-1 having antianaphylactic activity, stabilize antigen induced degranulation of mat cell and prolong the time of PCD. In conclusion, AHR-1 is might be effective in treatment various hypersensitivity reactions like anaphylactic shock and asthma.

REFERENCES

1. Ring J, Kramer U, Shafer T, Beherendt H. Why are allergies increasing? Curr Opinions Immunol, 2001; 13: 701–708.
2. Bousquet J, Bousquet PJ, Godard P, Daures JP. The public health implications of asthma. Bull World Health Organ, 2005; 83: 548–554.
3. Kelly HW, Sorknes CA. Asthma. *Pharmacotherapy – A Pathophysiological Aproch,* 6th ed. Dipiro JT, Talbert RL, Yee GC, Matzke TR, Wells BG, Posey LM. New York. The McGraw-Hill. 2005; p 504.
4. Kambayashi T, Koretzky GA. Proximal signaling events in FC£ RI mediated mast cell activation. J Allergy ClinImmunol, 2007; 119: 544–552.
5. Church MK, Levi-Schaffer F. The human mast cell. J Allergy ClinImmunol, 1997; 99: 155–160.
6. Salib RJ, Drake-Lee A, Howarth PH. Allergic rhinitis: past, present and future. *Clin Otolaryngol*. 2003; 28: 291–303.
7. Chakraborty A, Brantner AH. Study of alkaloids from *Adhatoda vasica* Nees. on their anti-inflammatory activity. Phytotherapy Res, 2001; 15: 532–534.
8. Dhuley JN. Antitussive effect of *Adhatoda vasica* extract on mechanical and chemical stimulation-induced coughing in animals. J Ethnopharmacol, 1999; 67: 361–365.
9. Amin AH, Mehta DR. A bronchodilator alkaloid (vasicinone) from *Adhatoda vasica* Nees. Nature, 1959; 184: 13–17.
10. Shashidhara S, Bhandarkar AV, Deepak M. Comparative evaluation of successive extracts of leaf and stem bark of *Albizzia lebbeck* for mast cell stabilization activity. Fitoterapia, 2008; 79(4): 301–302.
11. Babu NP, Pandikumar P, Ignacimuthu S. Anti-inflammatory activity of *Albizia lebbeck* Benth., an ethnomedicinal plant, in acute and chronic animal models of inflammation. J Ethnopharmacol, 2009; 125(2): 356–360.
12. Wu CN. Safety and anti-inflammatory activity of curcumin: A component of turmeric (*Curcuma longa*). J Altern Complement Med. 2003; 9: 161–168.
13. Palit G, Singh SP, Singh N, Kohli RP, Bhargava KP. An experimental evaluation of anti-asthmatic plant drugs from ancient Ayurvedic medicine. Aspects Aller Appl Immunol, 1983; 16: 36–41.
14. Anwikar S, Bhitre M. Study of the synergistic anti-inflammatory activity *of Solanum xanthocarpum*s chrad and Wendl and *Cassia fistula* Linn. Int J Ayurveda Res, 2010; 1(3): 167–171.

15. Bhitre J, Bhakti P, Milind. Study of the synergistic antiinflammatory activity of *Solanum xanthocarpum*s chrad and wendl and *Piper nigrum* linn. Int J Ayurvedic Herbal Med, 2011; 1: 42–53.
16. Keshavamurthy KR. *Medicinal plants of Karnataka.*1st *ed.* Karnataka Forest Department; 1994.
17. Dahanukar SA, Karandikar SM. Evaluation of anti-allergic activity of *Piper longum.* Indian Drugs, 1984; 21: 377–383.
18. Nair AM, Tamhankar CP, Saraf MN. Studies on the mast cell stabilizing activity of *Vitex negundo Linn*. Indian Drugs, 1995; 32: 277–282.
19. Gokhle AB, Dikshit VJ, Damle AS, Kulkarni KR, Saraf MN. Influence of ethanolic extract of *Tephrosia purpurea* Linn. on mast cells and erythrocytes membrane integrity. Ind J ExpBiol, 2000; 385: 837–840.
20. Gupta I, Gupta V, Parihar A, Gupta S, Lüdtke R, Safayhi H, et al. Effects of *Boswellia serrata* gum resin in patients with bronchial asthma: results of a double-blind, placebo-controlled, 6-week clinical study. Eur J Med Res, 1998; 3(11): 511–514.
21. Ammon HPT, Safayhi H, Mack T, Sabieraj J. Mechanism of antiinflammatory actions of curcumine and boswellic acids. J Ethnopharmacol, 1993; 38(2-3): 105–112.
22. Rawat A, Singh A, Singh AB, Gaur SN, Kumar L, Roy I et al. Clinical and immunologic evaluation of *Cedrus deodara* pollen: a new allergen from India. Allergy, 2000; *55*(7): 620–626.
23. Aggarwal BB, Sahdeo P, Simone R, Ramaswamy K, Vivek RY, Byoungduck P, et al. Identification of novel anti-inflammatory agents from Ayurvedic medicine for prevention of chronic diseases. Current drug targets, 2011; 12(11): 1595–1653.
24. Dorsch W, Stuppner H, Wagner H, Gropp M, Demoulin S, Ring J. Antiasthmatic effects of *Picrorhiza kurroa:* Androsin prevents allergen-and PAF-induced bronchial obstruction in guinea pigs. Int Arch of Allergy Immunol, 1991; 95: 128–133.
25. Nayampalli S, Ainapure SS, Nadkarni PM. Study of anti- allergic acid Bronchodilator effects of *Tinospora cordifolia*. Indian J Pharm, 1982; 14 : 64–66.
26. Udupa AL, Udupa SL, Guruswamy MN. The Possible Site of Anti-Asthmatic Action of *Tylophora asthmatica* on Pituitary-Adrenal Axis in Albino Rats. Planta Med, 1991; 57(5): 409–413.
27. OECD Guidelines for testing chemicals, Guidelines 425, Acute Oral Toxicity: Acute Toxic Class Method Paris, 2001.
28. Gupta SS, Paresh RM, Ram AK. Development of antihistamine and anti- allergic activity after prolonged administration of a plant saponin from *clerodendrum serratum*. J Pharmacy and Pharmacol, 1968; 20: 801–802.
29. Horn BR, Robin ED, Theodore J. Total eosinophils count in the management of bronchial asthma. New EnglJ Med, 1975; 292: 1152–1155.
30. Gopumadhavan S, Rafiq M, Venkataranganna MV, Mitra SK. Antihistaminic and antianaphylactic activity of HK-07, a herbal formulation. Indian J Pharmacol, 2005; 37(5): 300–303.
31. Patel PK, Patel KV, Gandhi TR. Evaluation of Effect of *Taxus baccata* Leaves Extract on Bronchoconstriction and Bronchial Hyperreactivity in Experimental Animals. J Young Pharm, *2011; 3(1): 41–47.*
32. Dale HH, Hartly P. Anaphylaxis to separated proteins of horse serum. Biochem J, 1916; 10: 408–433.
33. Humphrey JH, Mota I. The Mechanism of Anaphylaxis: Specificity of Antigen-Induced Mast Cell Damage in Anaphylaxis in the Guinea Pig. Immunology, 1959; 2(1): 31–43.
34. Nelson HS. Prospects for antihistamine in the treatment of asthma. J Allergy ClinImmunol. 2003; 112: S96–S100.
35. Bosquet J, Jeffery PK, Busse WW. Asthma: From bronchoconstriction to airway inflammation and remodeling. Am J Respi Care Med. 2000; 161: 1745–1749.

Pharmacological Evaluation of Cucumber for Cognition Enhancing Effect on Brain of Mice

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ABSTRACT

Introduction: Cucumber is fruit of *Cucumis sativus* var. *sativus* L. which has been used traditionally in gastrointestinal problems, skin problems and as coolant in salad for body and brain. Cucumber is a great folk medicine used to reduce heat and inflammation. Cognitive effects of cucumber are assessed in this study. **Methods:** Fresh fruits of *Cucumis sativus* L. were ground and a paste was prepared which consisted of different concentrations of cucumber (30, 60, 90 % w/w). The three doses were given *ad libitum* to mice for 15 successive days. Animal models utilized were sodium nitrite induced hypoxia and object recognition task. Biochemical analysis employed estimation of acetylcholinesterase activity in brain, serum glucose levels, cholesterol levels, brain lipid peroxidation (MDA) levels and reduced glutathione levels in brain of mice. **Results:** 6g/kg and 9g/kg doses of cucumber significantly ($P < 0.05$, $P < 0.01$) increased frequency of entry, number of entry and duration of entry in small compartment in sodium nitrite induced hypoxia model and depicted significantly ($P < 0.05$, $P < 0.001$) enhanced exploratory activity in object recognition task model. Further, biochemical analysis indicated good potential of cucumber in cognition enhancement. 9 g/kg dose ($P < 0.001$) reduced brain AChE activity along with blood glucose and serum cholesterol levels. 6 g/kg dose ($P < 0.01$) replenished brain GSH levels and reduced lipid peroxides. **Conclusion:** Cucumber increased cognition in rodents.

Keywords: Hypoxia, object recognition task, acetylcholinesterase, glucose, cholesterol, dementia

INTRODUCTION

Cucumber is a fruit of *Cucumis sativus* var. *sativus* L. (Cucurbitaceae). The plant is a creeping vine bearing cylindrical fruit which is thought to be one of the oldest vegetables cultivated by man with historical records dating back 5000 years back.[1] Traditionally cucumber has been used in diverse gastrointestinal problems[2] but now in light of its other chemical constituents it has a wide variety of applications such as hypolipidemic,[3] hypoglycemic,[4] anti-cancer,[5] hepatoprotective[6] and anti-inflammatory[7]. Different chemical compounds such as glycosides, many vitamins, sitosterol, bitter principle cucurbitacins, cucumegastigmanes, lactic acid, agmatine,[8] polyamines,[9] triterpenoids, polyphenols, flavonoids, many amino acids etc have been isolated.[10]

Cognition is the process by which the sensory input is transformed, reduced, elaborated, stored, recovered and used. Cognitive disorders are a category of mental health disorders that primarily affect learning, memory, perception and problem solving, ultimately leading to incapability in coping with complex and everyday activities. According to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) the three main areas of cognitive disorder include amnesia, dementia, and delirium.[11–12] Cognitive impairment is an important manifestation of several disorders such as affective disorders, schizophrenia, traumatic brain injury, cancer, infections, vascular diseases, ADHD etc. Alzheimer's disease (AD) is the most significant cause of cognitive impairment in adults.

In light of its traditional uses and recent studies indicating the presence of flavonoids, choline, agmatine and other polyamines in its seeds; it may possess brain function modulating activity.[8] In this study we have focused on one most critical domain of cognitive disorder i.e

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DOI: 10.5530/pj.2014.3.15

dementia, since delirium is temporary but dementia is progressive and unrelenting. The most common form of dementia in developed countries which primarily affects elderly people is Alzheimer's disease. In dementia the worst affected functions include personality, language, learning, memory and judgments.[11]

MATERIALS AND METHODS

Experimental design

Fresh cucumber was purchased from local market in Hisar, Haryana, India. The fruit was authenticated at Guru Jambheshwar University of Science and Technology, Hisar, Haryana. The fresh fruits of unpeeled cucumber were ground into a fine paste using an electric grinder. Different concentrations (30, 60, 90 % w/w) of cucumber paste in doses 3, 6 and 9 g/kg were fed *ad libitum* to separate groups of rodents through a specially prepared diet for 15 days successively. This special diet comprised of a mixture of cucumber paste, standard pellet diet (Ashirwad, Chandigarh, India) and a pinch of salt (sodium chloride); to impart taste. Each animal consumed around 5 g/day of this specially prepared diet. Control animals received the normal standard pellet diet without cucumber paste. On 14th day, 90 min after the administration of diet, the animals were exposed to object recognition task and sodium nitrite induced hypoxia. The retention of the learning was measured on 15th day after 24 h. After behavioral estimations; the mice were sacrificed to measure brain acetylcholinesterase, malonadialdehyde, blood glucose, serum cholesterol and brain reduced GSH levels. All the results were expressed as mean ± SEM. Data were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's *t* - test on Sigmastat. *P* value < 0.05 - 0.001 was considered to be significant. Karl Pearson's coefficient was determined for variables of parameters in object recognition task model.

Chemicals and vehicles used

5,5'-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholine iodide, eserine salicylate (Hi-Media, India), piracetam (UCB India Limited, India), simvastatin (Krabs Biochemicals and Industries Limited, India), donepezil (Merck, Germany), cholesterol diagnostics kit (Erba Diagnostics, Germany), cholinesterase diagnostics kit (Bayer Diagnostics, India). Donepezil injection and piracetam injection were dissolved separately in normal saline and injected intraperitoneally. Simvastatin was suspended with 0.5% w/v carboxymethylcellulose sodium and given orally. Volume of oral administration and intraperitoneal injection was 1 ml/100 g of mouse.

Animals

Swiss adult mice (5-9 months old) weighing around 28 g of either sex were procured from the Disease Free Small Animal House, CCS Haryana Agricultural University, Hisar (Haryana), India. The animals had free access to food and water, and were housed in an animal room with alternating light-dark cycle of 12 h each. The animals were acclimatized for at least 5 days to the laboratory conditions before behavioral experiments. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol and the care of laboratory animals was taken as per the guidelines of CPCSEA, Ministry of Forests and Environment, Government of India (registration number 0436).

Sodium nitrite induced metabolic hypoxia

The observation area consists of a rectangular box of dimension 35 cm x 20 cm x 25 cm divided into a small chamber (1/4th area) and a large one. Using lower doses of sodium nitrite (75 mg/kg, s.c.) mice were submitted to a positive reinforcement paradigm.[13] Groups of 6 mice were water deprived for 24 h. The mice were treated with the test compound 45 min before they are placed individually in a large chamber. On one wall of the chamber, there is a small compartment that contains a water bottle. The mouse easily finds the bottle and is allowed to drink for 30 s. Each mouse then received a subcutaneous injection of 75 mg/kg sodium nitrite ($NaNO_2$) before being returned to the home cage. Twenty-four hours later, retention testing was performed by placing the mouse in the large chamber, but at this time, the small compartment was kept empty. The frequency of the mouse exploration of the small compartment (FESC), number of entries of mouse in the small compartment (NESC), duration of exploration of the small compartment (DESC) while searching for water is evaluated over a period of 3 min. An increase in duration and frequency correlates with improved memory.

Object recognition task

The observation area consisted of a circular open field, 480 mm in diameter and the wall height 400 mm. Four different sets of objects, made of aluminum were used i) a cone ii) a ball iii) a plate iv) a glass bottle filled with sand. All objects were available in triplicate. They could not be displaced by the mouse nor could the mouse climb onto or hide under the objects. The objects had no natural significance and they were never associated with any kind of rein forcer. Two objects were presented in the first trial and a third one in the recognition trial to prevent odor

cues. Objects were cleaned with tab water and detergents after each trial. During two consecutive days, the mice were habituated to the apparatus and the testing procedure. They were allowed to explore the empty apparatus twice for 3 min each day (one morning and one afternoon session). Animal was placed into the apparatus, equidistant from the two objects, facing the wall in front of the experimenter. Duration of exploration was 3 min.

Animals were trained in pairs of two trials that were separated by a retention interval of one hour. During the first trial (T1) the apparatus contained two identical objects, "A1" and "A2". These objects were placed in a symmetrical position about 120 mm (with reference to the centre of the object) away from the wall. During T2 the apparatus contained two different objects, a copy of the familiar one "A" from T1 and a novel object "B". Mean time (in sec) exploring the familiar object A(a) and B(b) during T2; and A(a1) and A(a2) during T1 were measured. Following parameters were measured (Table 1)

Exploration is defined as follows: directing the nose to the object at a distance of no more than 2 cm and/or touching the object with nose. Sitting on the object is not considered as exploratory behavior.

Location preference was tested by comparing a1 and a2 (paired *t*-test). A virtual group was constructed with a mean of zero and SEM that corresponded with the average SEM of the discrimination parameter. This group can be considered as a group that did not discriminate between the objects with an associated expected sample variation. Although this can be considered as an arbitrary choice, it provides a most optimal manner to evaluate whether the discrimination performance of a group in a specific delay condition differed from zero. For this virtual group, the mean (SEM) calculated for d1 and d2 were 0 (1.37) and 0 (0.06) respectively. Values of d2 below 0.15 can be considered as failure to discriminate. This value will refer to as the 'discrimination level' in the present study. In addition, the interdependency of different measures of object recognition performance (e1, e2, d1 and d2) was assessed using Pearson's correlation coefficient (r_p).[14]

Collection of blood and brain samples

The animals were sacrificed by cervical decapitation under light anesthesia on the 15th day, 90 min after administration of the last dose of cucumber. Immediately after decapitation, the trunk blood was collected. Then whole brain was carefully removed from the skull. The collected blood was centrifuged at 3000 rpm for 15 min so as to separate the serum. The serum was used for estimation of total cholesterol levels. For preparation of brain homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of brain acetylcholinesterase activity.

Estimation of brain acetylcholinesterase

Brain acetylcholinesterase activity (AChE) was measured by the method of Ellman *et al.*[15–16] The change in absorbance per minute of the sample was measured on Double Beam UV-Visible spectrophotometer (Systronics 2203, Bangalore, India) at 420 nm.

Estimation of serum total cholesterol level

CHOD-PAP method by Allain *et al.*[17] was used for the estimation of serum total cholesterol. The absorbance was read at 510 nm and 630 nm (Filter 1 and Filter 2) against the blank sample by using double beam UV-Visible spectrophotometer (Systronics 2203, Bangalore, India).

Estimation of brain lipid peroxide level

Malondialdehyde (MDA), an index of free radical generation/lipid peroxidation, was determined as described by Ohkawa *et al.*[18] The MDA content was expressed

Table 1. Parameters analyzed in object recognition task (ORT)

Parameters analyzed	Index	Calculation
Exploration time for both objects during T1	e1	e1 = a1 + a2
Measure of global habituation from T1 to T2	h1	h1 = e1 – e2
Exploration time for both objects during T2	e2	e2 = a + b
Discrimination between familiar and novel object during T2	d1	d1 = b – a
Discrimination between familiar and novel object during T2, a relative measure corrected for explorative activity (e2)	d2	d2 = e2 / d1

as nmol/mg protein. The protein concentration was estimated by Lowry method using bovine serum albumin as the standard.[19] Absorbance was measured at 532 nm using double beam UV-Visible spectrophotometer (Systronics 2203, Bangalore, India).

Estimation of brain reduced glutathione levels

Absorbance was measured at 412 nm on double beam Double beam UV-Visible spectrophotometer. Data are expressed as μg per g wet weight of brain tissue.[20]

Estimation of blood glucose level

GOD-POD[21] method was used for the estimation of blood glucose. The absorbance was read at 510 nm and 630 nm (Filter 1 and Filter 2) against the blank sample by using Autoanalyzer (Erba Mannheim Chem-5 Plus V2).

RESULTS

Cucumber paste given to mice *ad libitum* has not affected the diet intake and weight of animals in comparison of control group animals.

Effect of cucumber on $NaNO_2$ induced metabolic hypoxia in mice

FESC is defined as the number of entries made by mice in small compartment per minute. NESC is defined as the number of entries made by mice in small compartment in 3 min time period. DESC is defined as the total time period for which the mice remain inside the small chamber in 3 min time period. The 6g/kg and 9g/kg dose of cucumber given orally for 15 successive days reversed the metabolic hypoxic condition created by $NaNO_2$, thereby significantly enhanced the memory of adult mice as compared to control group, by increasing FESC, NESC and DESC (Table 3).

Table 2. Pearson's correlation coefficients (r_p) between different measures of object recognition performance (e1, e2, d1 and d2) in adult mice. Values are in mean ± SEM. (n=6)

	e1	e2	d1
e2	0.152	–	–
d1	0.412	0.457	–
d2	0.426	0.125	0.930

Effect of cucumber on memory of mice using ORT

It can be seen that the habituation / familiarity (f) indices were negative. Negative value reflected an increase in the total exploratory activity from T1 to T2. The exploration times in T1 were found to be not significant as compared to control for any of three doses of cucumber. 9g/kg dose is the highest effective dose as compared to control group (Table 4). Results showed a significantly increased time taken by the mice for exploration of object a1 in trial 1 as compared to object a2 which depicts the location preference of mice for object exploration. However, in trial 2 the mice spends more time in exploring object b as compared to object a1. The object a1 was placed according to location preference of mice in trial 2 (Table 5).

The Pearson's coefficient depicted the sympathy among all the variables in this procedure. A positive value indicates a positive relationship between variables and a negative value indicates a negative relationship. It depicts whether the variables are in proportional to each other or not (Table 2).

Biochemical tests to identify metabolic effects of cucumber in mice

Results depicted significant decrease in acetylcholinesterase activity in brain of mice by 6g/kg and 9g/kg dose. All the three chosen doses reduced serum cholesterol levels, lipid peroxide levels in brain of mice and blood glucose levels, while these doses increased the brain GSH levels in adult mice (Table 6).

Table 3. Effect of cucumber on $NaNO_2$ induced metabolic hypoxia in adult mice

Treatment	Dose (kg^{-1})	FESC (min^{-1})	NESC	DESC (sec)
Control	Normal Diet	2.33 ± 0.21	7.00 ± 0.63	90.33 ± 1.20
$NaNO_2$	75 mg	0.5 ± 0.08*	1.67 ± 0.21*	72.50 ± 2.17#
$NaNO_2$ + Piracetam	75 mg + 400 mg	4.77 ± 0.48#	14.33 ± 1.43#	116.33 ± 2.73#
Piracetam	400 mg	5.22 ± 0.46#	15.67 ± 1.38#	125.83 ± 1.74#
$NaNO_2$ + CP	75 mg + 3 g	1.38 ± 0.16	4.17 ± 0.48	98.33 ± 2.09
$NaNO_2$ + CP	75 mg + 6 g	4.00 ± 0.51*	12.00 ± 1.53*	101.83 ± 1.92*
$NaNO_2$ + CP	75 mg + 9 g	4.61 ± 0.45•	13.83 ± 1.35•	103.17 ± 3.38•

Values were in mean ± SEM. Statistical significance was determined by one way ANOVA, followed by Dunnett's *t* - test. # $P < 0.001$, • $P < 0.01$, * $P < 0.05$. CP = Cucumber paste.

Table 4. Effect of cucumber on habituation (f) index, e1, e2, d1 and d2 values of mice

Treatment	Dose (kg^{-1})	f (sec)	e1 (sec)	e2 (sec)	d1 (sec)	d2 (sec)
Control	ND	–8.17 ± 1.62	9.67 ± 0.92	17.83 ± 1.35	3.33 ± 0.76	0.18 ± 0.03
Piracetam	400 mg	–5.67 ± 2.11	13.67 ± 0.99	19.33 ± 1.33	10.67 ± 0.21#	0.56 ± 0.03#
CP	3 g	–7.17 ± 1.19	12.17 ± 0.83	19.33 ± 0.614	6.67 ± 0.80*	0.35 ± 0.05*
CP	6 g	–8.67 ± 0.92	10.5 ± 0.81	19.17 ± 1.05	7.83 ± 0.98•	0.40 ± 0.04•
CP	9 g	–8.17 ± 1.28	12.67 ± 1.02	20.83 ± 1.40	10.5 ± 0.92#	0.50 ± 0.03#

Values were in mean ± SEM. Statistical significance was determined by one way ANOVA, followed by Dunnett's *t* - test. # $P < 0.001$, • $P < 0.01$, * $P < 0.05$. CP = Cucumber paste, ND = Normal diet. e2, F = 0.8031, p value is 0.5348 (NS); d1, F = 14.923; d2, F = 15.427. p value is <0.0001.

Table 5. Effect of cucumber on exploration of individual objects in trial 1 and trial 2 by mice. Paired *t* - test employed for comparison of a1 and a2 of trial 1

Treatment	Dose (kg^{-1})	a1 (sec)	a2 (sec)	a1 (sec)	b (sec)
Control	Normal Diet	6.33 ± 0.67#	3.33 ± 0.33	7.5 ± 0.67	10.83 ± 1.14
Piracetam	400 mg	9.0 ± 0.58 •	4.67 ± 0.42	4.33 ± 0.61	15.00 ± 0.73
CP	3 g	8.17 ± 0.60•	4.0 ± 0.36	6.33 ± 0.62	13.00 ± 0.36
CP	6 g	6.83 ± 0.60•	3.67 ± 0.33	5.67 ± 0.42	13.50 ± 0.92
CP	9 g	9.33 ± 0.98•	3.33 ± 0.21	5.17 ± 0.48	15.67 ± 1.08

Values were in mean ± SEM. Statistical significance was determined by one way ANOVA, followed by Dunnett's *t* - test. # $P < 0.001$, • $P < 0.01$, * $P < 0.05$. CP = Cucumber paste.

Table 6. Effect of cucumber on AchE levels, serum cholesterol levels, blood glucose, LPO (MDA) and brain GSH levels

Treatment	Dose (kg^{-1})	AChE activity (µmol/l/min/g tissue)	Serum Cholesterol (mg/dl)	LPO level (nmolMDA/ g wet wt.)	GSH levels (µmol/g wet wt.)	Blood Glucose (mg/dl)
Control	ND	16.10 ± 0.16	120.86 ± 5.35	114.56 ± 4.34	231.22 ± 12.86	240.40 ± 4.62
DNZ	0.1 mg	13.39 ± 0.34#	----------	----------	---------	---------
SMV	5 mg	----------	89.83 ± 2.81#	----------	---------	--------
CP	3 g	15.66 ± 0.19	105.58 ± 2.65*	102.58 ± 1.02*	277.24 ± 4.68*	220.41 ± 2.75*
CP	6 g	14.75 ± 0.26*	105.11 ± 3.70*	96.96 ± 2.69•	280.75 ± 8.17•	218.89 ± 6.23*
CP	9 g	14.33 ± 0.41•	102.28 ± 2.37•	92.42 ± 2.54#	309.27 ± 9.91#	216.62 ± 2.80•

Values were in mean ± SEM. Statistical significance was determined by one way ANOVA, followed by Dunnett's *t* - test. # $P < 0.001$, • $P < 0.01$, * $P < 0.05$. CP = Cucumber paste, ND=Normal diet, DNZ = Donepezil, SMV = Simvastatin. For brain AChE levels F = 13.989; for serum cholesterol levels F = 9.725, p value is <0.0001; for LPO levels F = 10.897, p value is 0.0002; for GSH levels F = 11.842, p value is <0.0001.

DISCUSSION AND CONCLUSION

Sodium nitrite induced brain hypoxia or metabolic hypoxia is an interoceptive behavioral model which can be utilized for screening of nootropics and free radical scavengers having memory improving effects. Reduction in oxygen supply to brain as in case of hypoxia, hypercapnia or ischemia is reported to produce amnesia in rodents. Sodium nitrite administered to mice after training elicited retrograde amnesia in mice. Our results reflected significant increase in scores of FESC, NESC and DESC as compared to control groups in mice by cucumber administered 15 days successively, which indicated a vivid and consummate reversal of sodium nitrite induced retrograde amnesia in mice.

Sodium nitrite releases nitric oxide (NO) *in vivo*. Biosynthesis of NO occurs throughout the CNS particularly in the cerebellum in neurons as well as in glia from L-arginine by nitric oxide synthase.[22] Nitric oxide plays essential roles in neurotransmission and memory.[23] Normally N-methyl D-aspartate receptors are associated with synaptic plasticity and memory functions. The activation of N-methyl D-aspartic acid (NMDA) receptors leads to increased concentration of calcium ions intracellularly which begets activation of nitric oxide synthase (NOS) activity. Surfeit of NO in brain leads to degeneration of neurons due to exictotoxicity[24] and free radicals [nitrite and peroxynitrite ($ONOO^-$) anions] generated by reaction of oxygen with NO. Cytokines originated from microglia may cause production of inducible nitric oxide synthase

(iNOS) and hence excess NO by microglia. Nitric oxide also binds to cytochrome C oxidase in mitochondria and is able to fetter cell respiration in a process that is reversible and in competition with oxygen.[25] This action can also lead to the release of superoxide anion from the mitochondrial respiratory chain. Thus, according to our results in pathophysiological conditions such as brain ischemia or neurological disorders, cucumber might have precluded the over-excitation of NMDA receptors. Furthermore, according to literature agmatine might inhibit the production of NO by proscribing the NOS instigation. Moreover, cucumber might scavenge the free radicals or curtailed their production and promoted the cell respiration.

Free radicals are the chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive brain tissue damage which can lead to cognitive dysfunction.[26–27] Cucumber reduces oxidative stress by enhancing the levels of GSH in mice. Oxidative stress can damage neurons and promote the release of excitatory amino acids, generating a 'vicious cycle' of events. Several neurotransmitters (not glycine or glutamate) are autoxidizable. Dopamine, its precursor L-DOPA, serotonin and norepinephrine can react with O_2 to generate not only O^{-2} but also quinones/semiquinones that can deplete reduced glutathione (GSH) and bind to protein SH groups.[28] Cucumber may fetter the oxidation of neurotransmitters. Normal brain also generates H_2O_2 via enzyme catalyzation by MAO A and B, SOD, flavoprotein enzymes located in outer mitochondrial membrane.[29] Agmatine deactivates the MAO A and B enzymes through I_2-receptor modulation. The rise in arachidonic acid coupled with increased lipid peroxidation can promote eicosanoid formation[30] and, if prostaglandins are not quickly removed, they can undergo conversion to neurotoxic agents, the cyclopentenone prostaglandins and the levuglandins.[31]

A number of studies confirmed that the redox status of intracellular reduced glutathione (GSH) – the major antioxidant thiol in mammalian cells – would be a critical factor in determining cellular susceptibility to peroxynitrite. Thus, glutathione deficiency facilitates NO and peroxynitrite-dependent neurotoxicity by, possibly, increasing the rate of protein nitration and mitochondrial damage at complex I.[32] The mitochondrial content of glutathione is especially relevant for protecting the brain against peroxynitrite-mediated cellular damage.[33] The most important H_2O_2- removing enzymes in brain and other animal tissues are the glutathione peroxidases (GPx), a family of selenium-containing enzymes. They remove H_2O_2 by coupling its reduction to H_2O_2 with the oxidation of GSH, a thiol-containing tripeptide (Glu-Cys-Gly). Reduced GSH protects cells from the toxic effect of reactive oxygen species or peroxidative damage and contributes to the elimination of organic peroxides and foreign compounds. It is also involved in the preservation of thiol disulfide status of protein.[34] The regeneration of reduced vitamin E (inhibits chain propagation reaction) from oxidized form is essential for antioxidant activity, which requires GSH. Our findings indicated that cucumber doses culminated in a cogent way to significant increase in GSH levels.

Object recognition task is a one-trial learning task having several advantages for assessing the effects of drug treatment on memory processes independently i.e. acquisition, consolidation and retrieval. In the present study, the cucumber significantly enhanced the absolute discrimination index d2 and relative discrimination index d1 as compared to control groups which reflected a remarkable increase in the recognition of new object by mice in trial T2. Cucumber also protracted the exploratory activity of mice significantly in trial T2 as compared to control group, which manifested perspicuously increased ability of mice for object differentiation. In general it is assumed that d2 is more reliable measure of discrimination performance than the d1 because it corrects for the total exploratory activity. But it has to be noted that also d2 should be treated with caution, as can be inferred from our data. A small change in exploration time in T2 will have a large effect on d2 value. But if total exploration is high enough then it is less likely to affect the d2 value i.e. d2 will then be independent of e2.

Release of acetylcholine in amygdale positively correlates with the performance on hippocampus-dependent task.[35] In the present study cucumber administered for 14 days to mice showed significant reduction of brain acetyl-cholinesterase activity thereby probably facilitating cholinergic transmission by replenish the stores of Ach and improving memory of animals. Furthermore, choline is present in high amounts in cucumber which also enhance the Ach transmission in brain by providing precursor molecule for its synthesis.

Epidemiological studies indicates that high level of cholesterol contribute to the pathogenesis of cognitive dysfunction.[36] Interestingly, the mice which were treated with cucumber showed significant reduction in cholesterol levels in as compared to control group.

Hyperglycemia imparts deleterious effect on the cognition of rodents.[37] Cucumber was found to reduce the blood glucose levels in present study. The hypoglycemic action of cucumber may be due to its extrapancreatic site of action, that is, by direct metabolic effect on tissues, particularly liver.[38] Insulin and its receptors are widely distributed throughout the brain, with particular abundance in defined areas, such as the hypothalamus and the hippocampus. Insulin may promote glucose utilization in hypothalamus and hippocampal regions thereby increase the memory.[39] Due to hypoglycemic activity cucumber also prevented sensory neurons, satellite cells, and Schwann cells in dorsal root ganglia of spinal nerves, which may undergo apoptosis in culture with high glucose concentration (>50 mM) as reported in recent findings.[40]

CONCLUSION

In nutshell, cucumber improved the cognitive functions of rodents. Cucumber inhibited activity of cholinesterase enzyme in brain, reduced the blood glucose and serum cholesterol levels, deterred the lipid peroxidation in brain and replenished the GSH levels in brains of rodents. The properties possessed by cucumber culminate in prevention of neurodegenerative diseases vividly. Moreover, its potential to vitalize the neurons in ischemic conditions in perspicuous manner may propel its importance in brain injury, environmental toxicity and other hypoxic conditions.

CONFLICTS OF INTEREST

Both authors have none.

ACKNOWLEDGEMENT

The authors are grateful to Guru Jambheshwar University of Science and Technology, Hisar, Haryana, India for financial support and technical assistance.

REFERENCES

1. Eifediyi EK, Remlson SU. Growth and yield of cucumber (*Cucumis sativus*) as influenced by farmyard manure and inorganic fertilizer. J Plant Breed Crop Sci. 2010; 2(7): 216–20.
2. Enslin PR. Bitter Principles of the Cucurbitaceae I. Observation on the Chemistry of Cucurbitacin A. J Sci Food Agric. 1954; 5: 410–16.
3. Sudheesh S, Vijayalakshmi NR. Lipid-lowering action of pectin from *Cucumis sativus*. Food chem. 1999; 67(3): 281–86.
4. Roman- Ramos R, Flores-Saez JL, Alarcon-Aguilar FJ. Anti-hyperglycaemic effect of some edible plants. J Ethnopharmacol. 1995; 48(1): 25–32.
5. Rodriguez N, Vasquez Y, Hussein AA, Coley PD, Solis PN, Gupta MP. Cytotoxic cucurbitacin constituents from *Sloanea zuliaensis*. J Nat Prod. 2003; 66: 1515–1516.
6. Ahmad A, Manuel M, Jose J, Jose A, Dolores-Caracuel M, Garcia-Granados M, Navarro MC. Isolation of anti-hepatotoxic principles from the juice of *Ecballium elaterium*. Planta Med. 1999; 65: 73.
7. Recio MC, Prieto M, Bonucelli M, Orsi C, Manez S, Giner RM, Cerda-Nicolas M, Rios JL. Anti-inflammatory activity of two cucurbitacins isolated from *Cayaponia tayuya* roots. Planta Medica. 2004; 70: 414–1420.
8. Sabakibara Y, Yanagisawa H. Agmatine deaminase from cucumber seedlings is a mono-specific enzyme: Purification and Characteristics. Protein Expr Purif. 2003; 30(1): 88–93.
9. Hajar MA. 1,3-Diaminopropane and spermidine in *C. sativus* (cucumber). Phytochem. 1984; 23(5): 989–90.
10. Horie H. Cucurbitacin C- Bitter Principle in Cucumber Plants. JARQ. 2007; 41(1): 65–68.
11. Berryhill ME, Peterson D, Jones K, Tanoue R. Cognitive disorders. In: Ramchandran VS, Editor. Encyclopedia of Human Behavior. Elsevier, 2012; p. 536–42.
12. Ravdin LD. Cognitive disorders. In: Heggenbougen K, Quah S, Editors. International Encyclopedia of Public Health. Elsevier, 2008; p. 759–65.
13. Schindler U, Rush DK, Fielding S. Nootropic drugs: Animal models for studying effects on cognition. Drug Develop Res. 1984; 4: 567–76.
14. Sik A, Nieuwehuyzen PV, Prickaerts J, Blokland A. Performance of different mouse strains in an object recognition task. Behav Brain res. 2003; 147: 49–54.
15. Ellman GL, Courtney DK, Andres V, Feathestone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7: 88–95.
16. Voss G, Sachsse K. Red cell and plasma cholinesterase activities in micro samples of human and animal blood determined simultaneously by a modified acetylthiocholine/ DTNB procedure. Toxicol Appl Pharm. 1970; 16: 76472.
17. Allain CC, Poon LS, Chan CSG, Richmond W, Paul CF. Enzymatic determination of total serum cholesterol. Clin Chem. 1974; 20: 470–75.
18. Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95: 351–58.
19. Lowry OH, Rosdrough NJ, Randall R. Protein measurement with Folin phenol reagent. J Biol Chem. 1951; 193: 265–75.
20. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 1968; 25: 192–205.
21. Miksch R, Wiedemann G. Blood sugar determination with the GOD- POD-ABTS method using uranylactate for deproteinization. Z Med Labortech. 1973; 14(1): 27–33.
21. Cunningham C, Wilcockson DC, Campion S, Lunnon K, Perry VH. Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. J Neurosci. 2005; 25: 9275–9284.
23. Garthwaite J, Charles SL, Chess-Williams R. Endothelium derived relaxing factor release on activation of NMDA receptors suggests a role as intercellular messenger in the brain. Nature. 1988; 336: 385–87.
24. Mander P, Borutaite V, Moncada S, Brown GC. Nitric oxide from inflammatory-activated glia synergizes with hypoxia to induce neuronal death. J Neurosci Res. 2005; 79: 208–15.
25. Duncan AJ, Heales SJ. Nitric oxide and neurological disorders. Mol Aspects Med. 2005;26:67–96.
26. Berr C. Oxidative stress and cognitive impairment in the elderly. J Nutr Health Aging. 2002; 6: 261–266.

27. Perry G, Cash AD, Smith MA. Alzheimer disease and oxidative stress. J Biomed Biotech. 2002; 2: 120–23.
28. Wrona MZ, Dryhurst G. Oxidation of serotonin by superoxide radical: implications to neurodegenerative brain disorders. Chem Res Toxicol. 1998; 11: 639–50.
29. Gal S, Zheng H, Fridkin M, Youdim MB. Novel multifunctional neuroprotective iron chelator-monoamine oxidase inhibitor drugs for neurodegenerative diseases. *In vivo* selective brain monoamine oxidase inhibition and prevention of MPTP-induced striatal dopamine depletion. J Neurochem. 2005; 95: 79–88.
30. Phillis JW, O'Regan MH. A potentially critical role of phospholipases in central nervous system ischemic, traumatic, and neurodegenerative disorders. Brain Res Brain Res Rev. 2004; 44: 13–47.
31. Musiek ES, Milne GL, McLaughlin B, Morrow JD. Cyclopentenone eicosanoids as mediators of neurodegeneration: a pathogenic mechanism of oxidative stress-mediated and cyclooxygenase mediated neurotoxicity. Brain Pathol. 2005; 15: 149–58.
32. Hsu M, Srinivas B, Kumar J, Subramanian R, Andersen J. Glutathione depletion resulting in selective mitochondrial complex I inhibition in dopaminergic cells is via an NO-mediated pathway not involving peroxynitrite: implications for Parkinson's disease. J Neurochem. 2005; 92: 1091–1103.
33. Sims NR, Nilsson M, Muyderman H. Mitochondrial glutathione: a modulator of brain cell death. J Bioenerg Biomembr. 2004; 36: 329–33.
34. Shan XQ, Jones DP. Glutathione dependent protection against oxidative injury. Pharmacol Ther. 1990; 47: 61–71.
35. Agnolli A, Martucci N, Manna V, Conti L. Effect of cholinergic and anticholinergic drugs in short term memory in electroencephalographic study. Clin Neuropharmacol. 1983; 6: 311–23.
36. Koudinov AR, Koudinova NV. Brain cholesterol pathology is the cause of Alzheimer's Disease. Clin Med Health Res. 2001; 5: 1–6.
37. Gispen WH, Biessels G J. Cognation and synaptic plasticity in diabetes mellitus. Trends Neurosci. 2000; 23: 542–49.
38. Sharma MK, Khare AK, Feroz H. Effect of neem oil on blood sugar levels of normal, hyperglycemic and diabetic animals. Indian Med Gaz. 1983; 8*:* 380–83.
39. Dhingra D, Parle M, Kulkarni SK. Effect of combination of insulin with dextrose, D (–) fructose and diet on learning and memory in mice. Ind J of Pharmacol. 2003; 35: 151–56.
40. Delaney CL, Russell JW, Cheng H-L, Feldman EL. Insulin-like growth factor-I and over-expression of Bcl-xL prevent glucose-mediated apoptosis in Schwann cells. J Neuropath Exp Neur. 2001; 60(2): 147–60.

Role of *Boswellia ovalifoliolata* Bal. Henry extract on high fat diet induced hypercholesterolemia

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ABSTRACT

Objective: To evaluate the Antihypercholesterolemic effect of *Boswellia ovalifoliolata* Bal. Henry extract by performing invivo studies and to checkout its effects by evaluating parameters like food consumption, weight gain, fecal fat excretion, serum and liver lipid &biochemical profiles. Even the study includes confirmation of activity by the histopathological studies. **Methods:** Animals were fed with cholesterol rich high fat diet. Food intake, Bodyweight and fecal fat excretion were measured. Serum and liver samples were analyzed for the estimation of lipid profiles and other biochemical parameters by using different kits. Histopathological study on liver, aorta, heart and adipose tissue was done to ensure the activity. **Results:** The animal group administered with methanolic extract of the plant has shown decreased levels of TC, LDL, VLDL, TG, HDL+VLDL, VLDL+LDL, LDL/TC, AI, SGOT, SGPT and elevated levels of HDL, HDL/TC in a dose dependent manner significantly ($p<0.01$ & $p<0.05$). The evaluation of liver tissue of animal groups treated with herbal extract and standard had shown increased levels of SOD, GSH and Catalase, whereas levels of SGOT, SGPT, Total glucose, HMG-CoA, lipase, amylase and percentage of monaldehyde were decreased when compared with high fat diet fed rats. Body weight and Food intake in treated groups were significantly lower than that in model control. **Conclusion:** It can be confered from the present studies that the *Boswellia ovalifoliolata* Bal. Henry extract have strong activity against hypercholesterolemia and obesity suggesting a potential benefit as antihypercholesterolemic agent.

keywords: *Boswellia ovalifoliolata* Bal. Henry, High fat diet, Lipid profile, Histopathological studies

INTRODUCTION

Hypercholesteremia, a known risk factor is considered to be one of the reasons for cardiovascular disease (CAD) and hence as a major cause of premature death globally in many developing and developed countries like India[1] and the most European countries cardiovascular disease contributes about 40% to all-cause mortality.[2] It is estimated by World Health Organization that approximately one third of all cardiovascular disease worldwide were caused by high cholesterol, and in the USA, 105 million people have cholesterol levels to a cardiovascular risk.[3] Hyperlipidemia is characterized by elevated serum TC, LDL, VLDL and decreased HDL levels. Hyperlipidemia associated lipid disorders are found to be responsible for CAD[4] of which hypercholesterolemia and hypertriglyceridemia are closely related to ischemic heart disease.[5,6] The main aim of treatment in patients with hyperlipidemia is to reduce the risk of developing ischemic heart disease or the occurrence of further cardiovascular or cerebrovascular disease.[7] The treatment of hyperlipidemia involves synthetic hypolipidemic drugs[8] whose consumption may lead to hyperuricemia, diarrhoea, nausea, myositis, gastric irritation, flushing, dry skin and abnormal liver function[9] which consumption the traditional systems which have immune potential against various diseases. Medicinal plants are used for various research purposes. More than thirteen thousand plants have been studied for various pharmacological properties. Herbal treatment for

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DOI: 10.5530/pj.2014.3.16

hypercholesterolemia has been associated with fewer side effects and is relatively cheap, locally available and they are effective in reducing the lipid levels in the system.[10] Hyperlipidemia is classified as primary or secondary based on complexities associated with disease of which anti-lipidemic drugs are used to treat primary disease, but the secondary type originating from diabetes, renal lipid nephrosis or hypothyroidism requires the treatment of the actual disease condition rather than simple hyperlipidemia based treatment.[11] Increased LDL formed from VLDL due to high fat consumption that adhere to walls of the blood vessels can block the normal blood flow resulting in the risk which can be prevented by improving the human diet which is highly recommended.[12] The lipid lowering action of the leaves of medicinal plants which play a major role in hypolipidemic activity is found to be mediated through the inhibition of the hepatic cholesterol biosynthesis and reduction of lipid absorption in the intestine. *Boswellia ovalifoliolata* Bal. Henry (Burseraceae) is distributed throughout hot spot of India. The plant is over exploited for its medicinal uses. The fresh leaf juice used to prevent throat ulcers. The stem is used in stomach ulcers and diabetes. Gum is used in dysentery, inflammations, joint pains, ulcers, arthritis and amoebic dysentery.[13] Decoction of the stem bark 10 – 25 mL per day reduces rheumatic pains. The gum obtained from the trunk which is highly medicated. Small lumps of fresh light yellow coloured liquid oozes out from the stem and hardens on exposure. Amyrins are the chief constituents of the gum together with resin acids and volatile acids. Shade dried gum is powdered dissolved in water and mixed with curd and given orally to cure amoebic dysentery.[14] Considering the traditional uses of the plant, the present study was focused on the effect of extract of plant on serum and liver lipid and other biochemical level in high fat diet fed sprague-dawley rats.

MATERIALS AND METHODS

Plant Material

The plant was collected from the surrounding areas of Talagona A.P, India, during November to February. The plant was identified by Botanist, in S.V.University. The plant was identified as *Boswellia ovalifoliolata* Bal. Henry belonging to family – Burseraceae.

Chemicals and Reagents

The solvent used for extraction was methanol. Other reagents used were obtained from various commercial sources are of laboratory and analytical grade. All diagnostic kits purchased from the Reckon diagnostic kit, India. All the parameters were estimated using an automatic analyzer (Robonik Touch, version 2.622A)

Preparation of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry (BOI)

The shade dried plant as a whole was powdered of which 50g was suspended and extracted with 10 volumes of methanol by shaking at room temperature for 15 hours, filtered through filter paper, and the supernatants were pooled. The residue was re-extracted under the same conditions. Pooled extracts were condensed (methanol was removed) by a rotary evaporator. The physical characteristics and percentage yield of methanol extracts were reported. The dried extracts of all solvent were kept in desiccators prior to analysis.[15]

Experimental Animals

Spraque Dawley rats (5 weeks old) purchased from the Sapthagiri Lab (India) were housed in stainless steel wire-bottomed cages under a 12-h light/12-h dark cycle in a temperature- and humidity-controlled room. They were allowed food and water ad libitum. After a 1 week time period for adaptation to lighting conditions, the healthy animals were used for experimentation. Rat weights at the beginning of the study ranged from 95 to 120 g. The doses of BOI were selected as per literature review[16] and 1% tween 80 used as a vehicle. Rat chow diet was supplied by Vyas labs (India). Proximate analysis of rat chow diet did show that it contains crude protein 21.3; crude fat 4.10; crude fiber 5.00; carbohydrate 53; total ash 7.4; and minerals and trace elements 5.34% along with other minerals and trace elements like copper 3.20; zinc 35.96; manganese 34.96; and cobalt 0.58mg/100g. Vitamins included A 56; D3 0.169; E 0.404; and B complex 5.073. To the powdered rat chow diet, deoxy cholic acid was added at a ratio of 5 gm for 700gm diet and thoroughly mixed. Simultaneously, cholesterol (5g) was dissolved in 300g warm coconut oil. Oil solution was added slowly into powdered mixture of above to obtain homogeneous soft cake. This cholesterol rich (HFD) preparation was molded in the shape of pellets of about 3g each.[17] The energy level of the prepared high-fat diet(HFD) was approximately 4996 kcal/kg, whereas rat chow diet was 3030 kcal/kg.

Experimental procedure

The rats were randomly divided in to 5 groups with 6 animals in each. Throughout the experimental period of 35 days, Group I, the normal control (NC) group was fed with normal rat chow diet, Group II (MC) with only

HFD, Group III(STD) was treated with HFD along with standard Atorvastatin (10 mg/Kg), Group IV and V were administered with HFD along with BOI 50 mg/kg and 100 mg/kg respectively. The groups II to V were fed with high fat diet to increase the serum lipid levels before the administration of herbal extract and standard for one week. Dose administration through oral route was started from the 7th day. The animal studies were performed in compliance with protocols and policies approved by the Institutional Animal Ethical Committee of Nalanda College of Pharmacy (NCOP), Nalgonda, India (Voucher no: NCOP/IAEC/Approval/22/2010). Animals were observed daily for any abnormal physical and behavioral changes or signs of toxicity. Food intake (FT), Body Weight (BW), mean food efficiency ratios and fecal fat excretion mass were done for this studies. At the end of the experiment, the rats were fasted for 12 hr and the blood was withdrawn from retro-orbital plexus of rat under the anesthesia which was centrifuged immediately at 3000rpm for 15mins to get the plasma samples and the samples thus obtained were analyzed for the estimation of lipid profiles like TG, TC, HDL & LDL, and other biochemical parameters like glucose, SGOT, SGPT, amylase, lipase, catalase, and TBARS by using diagnostic kits. VLDL, LDL, ratios of HDL/TC, AI and HDL/LDL were calculated to describe serum lipid levels. The liver was immediately removed and stored at deep freeze condition until analyzed. Liver tissues were minced and homogenized (10% w/v) in 0.1M phosphate buffer (pH 7.4). A part of homogenized solution was extracted with chloroform–methanol (2:1, v/v, 2 ml). The residue was analyzed with a TG, TC, HDL, LDL kit. Remaining part of homogenized solution was centrifuged at 5000 × g for 10 min and the resulting supernatant was used for analysis of SGOT, SGPT, Glucose, Lipase and amylase (using diagnostic kit), catalase, SOD, GSH and TBARS activity. For HMG-CoA/mevalonate ratio activity, liver tissue was homogenized in Saline arsenate solution. The remaining liver (after the experiment of hepatic lipid profile), heart, thoracic aorta and adipose tissues were isolated, cleaned and then fixed in a buffer solution of 10% neutral buffered formalin. For the histopathological studies, longitudinal sections of the myocardial tissue, adipose tissue and thoracic aorta were taken at the macroscopic lesions and the liver sections were cut through the macroscopic lesions including capsules. The sections were further cut to 5 µm thickness and were stained with H&E.

Statistical analysis

All the data was subjected to ANOVA (Graph pad Instat Demo software version 3.10). The data shown are mean value and the significance differences was compared by using Dunnett Multiple comparison test at the $P < 0.01$ probability level.

RESULTS

Food intake and Body weight (BW)

The BW of the rat in the NC group gradually increased during the 5-week period. In contrast, the BW of animals fed with HFD showed rapid increase whereas those fed with the HFD and herbal extracts showed a gradual increase in BW which was significantly less than that of the HFD control in spite of continued and prolonged access to the high-fat diet. Although rats fed with the normal and HF diet continued to show increased BW and FT until the end of the study, % BW reduction was 11.77% for BOI 50; 16.27% for BOI 100 and 38.41% ($p<0.01$) for STD. Percentage reduction in FT was 5.14%, 6.06% and 5.91% for BOI 50, BOI 100 and STD and % food efficiency ratio was 92.81%, 88.81% and 65.28% ($p<0.01$) for BOI 50, BOI 100 and STD. The loss of weight in extract treated groups resulted in the massive loss of body lipid and the preservation of proteins, thus increasing the proportion of latter and decreasing that of lipid. The HF diet groups with or without treatment of BOI did not cause diarrhea during the experiment. Food efficiency was increased in the HF group compared with the normal group, but treatment of BOI reduced the food efficiency. BW and food efficiency reduced significantly in atorvastatin treated group than model control group. These results suggest that BOI may prevent an increase of BW induced by a high fat diet; it seemed that low BW in herbal treated groups partially may be due to the loss of appetite. In order to understand change of appetite by ingesting herbal extracts, further research is to be done.

Fecal fat excretion

Fecal dry weight in the MC group was significantly higher than other groups. Fecal excretion of fat in the standard treated group was significantly higher than other groups. It can be considered that Atorvastatin reduce the absorption of cholesterol in the intestine and enhances the excretion of cholesterol. BOI treated groups also increased fecal excretion of fat in a dose dependent manner ($p<0.05$) and the effect was slightly less than that of standard treated group but more than control groups. Fecal excretion of fat was inversely proportional with serum cholesterol and liver cholesterol.

Serum Analysis

The lipid profile of the serum (TC, LDL, VLDL, TG, HDL+VLDL, VLDL+LDL, LDL/TC, HDL, HDL/TC & AI) and other biochemical parameters like SGOT and SGPT of rats from all groups was summarized in table 1. The MC group showed markedly higher serum TC, LDL, VLDL, TG, HDL+VLDL, VLDL+LDL, LDL/TC & AI levels and lower HDL & HDL/TC levels than the normal control group. Compared with HFD group, in the BOI treated groups TC, LDL, VLDL, TG, HDL+VLDL, VLDL+LDL, LDL/TC & AI were decreased significantly ($p<0.01$) and HDL & HDL/TC was increased significantly ($p<0.01$) in a dose dependent manner. These effects may be due to low activity of cholesterol biosynthesis enzymes and low level of lipolysis. The BOI extracts supplementation also results the significant attenuation in the level of serum HDL toward the control level which again strengthens the hypolipidemic effect of the extract. Animals treated with atorvastatin, the standard showed better results than the BOI treated groups in all lipid parameters. The content of HDL in serum implies the activity of LCAT, which plays a key role in lipoprotein metabolism and may contribute to the regulation of blood lipids. HDL/TC represents the proportion of cholesterol component and may provide valid indices for identifying individuals at risk of peripheral arterial diseases. Constituents present in BOI extracts may decrease the risk of cardiovascular disease by increasing the ratios. The HDL/TC ratio may hasten removal of cholesterol from peripheral tissues to liver for catabolism and excretion. SGOT, SGPT, plasma glucose, % MDA level, amylase and lipase levels were increased and catalase activity was decreased in the MC group. SGOT, SGPT, plasma glucose, amylase, lipase and % MDA(TBARS) were decreased significantly ($p<0.05$) and catalase activity in plasma was increased in BOI treated groups in a dose dependent manner than MC group after 35th day treatment. Plasma glucose reduction in BOI treated groups indicated that the BOI extract decreases hyperglycemia in obese rats. In the high-fat-diet control, there was little increment in glucose though it is within the range of 77-150mg/dl, where as in the BOI treated animals the determined levels were close to the normal range of glucose level. SGOT and SGPT values were applied to evaluate liver damage in the present study.

Table 1. the data of serum analysis

	TC (mg/dl)	TG (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)	HDL+VLDL (mg/dl)	LDL (mg/dl)	VLDL + LDL (mg/dl)	AI	HDL/TC (mg/dl)	LDL/TC
Normal control	44.90 ± 6.25**	39.39 ± 6.91**	7.87 ± 1.38**	22.24 ± 2.31	30.12 ± 3.66	14.78 ± 2.71**	22.66 ± 4.07**	1.00 ± 0.10*	0.50 ± 0.02**	0.32 ± 0.02**
model control	167.94 ± 5.24	148.01 ± 6.14	29.60 ± 1.22	31.93 ± 6.33	61.54 ± 7.36	106.39 ± 2.16	136 ± 1.46	4.72 ± 1.17	0.18 ± 0.03	0.63 ± 0.03
BOI 50	123.611 ± 7.225*	103.613 ± 4.124	20.723 ± 0.825	38.424 ± 0.815	59.147 ± 1.557	64.464 ± 6.571**	85.187 ± 7.098**	2.218 ± 0.188	0.313 ± 0.017	0.519 ± 0.023
BOI 100	111.690 ± 4.702**	87.063 ± 5.663*	17.413 ± 1.133*	37.515 ± 1.152	54.928 ± 1.474	56.762 ± 4.035**	74.175 ± 3.558**	1.975 ± 0.036	0.336 ± 0.004	0.507 ± 0.017
STD 10	97.22 ± 7.87**	83.68 ± 1.11*	16.73 ± 0.22*	43.63 ± 1.21	60.37 ± 1.34	36.84 ± 6.53**	53.58 ± 6.67**	1.22 ± 0.12*	0.45 ± 0.02**	0.37 ± 0.03**
	SGOT (IU/L)	**SGPT (IU/L)**	**Total glucose (mg/dl)**	**Amylase activity (IU/L)**	**Amylase inhibition (%)**	**Lipase (IU/L)**	**Lipase inhibition (%)**	**Catalase (µmoles/min/ml)**	**%MDA**	**% inhibition (%)**
Normal control	9.753 ± 1.352**	12.379 ± 0.65**	47.599 ± 1.909**	69.461 ± 2.989**	7.459 ± 3.982**	185.714 ± 25.754	23.527 ± 10.605	0.014 ± 0.007*	13.826 ± 0.137**	
model control	28.884 ± 0.375	31.509 ± 1.949	110.681 ± 1.056	75.069 ± 0.362**	–0.012 ± 0.482**	242.857 ± 18.898	–0.003 ± 7.782	0.004 ± 0	38.604 ± 1.442	–0.010 ± 3.736
BOI 50	25.508 ± 2.705	28.133 ± 5.664	82.939 ± 6.397*	44.137 ± 7.554*	41.198 ± 10.064*	135.714 ± 7.143	44.116 ± 2.941	0.018 ± 0**	28.063 ± 0.274*	27.298 ± 0.709*
BOI 100	21.756 ± 1.352	21.756 ± 1.352*	79.211 ± 0.313**	42.147 ± 5.393*	43.849 ± 7.185*	128.571 ± 12.372	47.057 ± 5.094	0.022 ± 0.001**	27.105 ± 1.034**	29.781 ± 2.678*
STD 10	13.879 ± 0.375*	15.755 ± 0.65**	64.946 ± 1.075**	19.536 ± 0.829	73.973 ± 1.104	121.429 ± 25.754	49.999 ± 10.605	0.034 ± 0.001**	29.295 ± 4.177*	24.106 ± 10.821*

Values are in mean ± SEM; Number of animals in each group =6; **P<0.01& *P<0.05 Vs Normal Control; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD 10 = treated with Atorvastatin (10mg/Kg).

Liver profile

In liver tissue, HDL, HDL/TC, SOD, GSH and Catalase were decreased and TC, TG, VLDL, HDL/VLDL, SGOT, SGPT, Total glucose, lipase, amylase, HMG-CoA/mevalonate ratio and % of monaldehyde were increased in high fat diet rats as compared to normal rats. In liver, HDL ($p<0.05$), HDL/TC, SOD, GSH, HMG-CoA/mevalonate ratio and Catalase were increased with supplementation of BOI extracts and standards, whereas TC ($p<0.05$), TG, VLDL, HDL/VLDL, SGOT, SGPT, Total glucose ($p<0.01$), lipase, amylase, and % of MDA were decreased as compared to model control (high fat diet rats) in dose dependent manner. Biochemical profile in liver of rats from all groups was summarized in table 2. In the groups treated with BOI extracts and standard, the BW and liver triacylglycerol level were reduced significantly when compared with high-fat diet-fed group. The increase in the intracellular deposition of TG in liver is well documented and demonstrated to attenuate glucose metabolism by interfering with insulin signaling and insulin secretion. Theoretically, Lipase inhibitors should inhibit fat accumulation in adipose tissue. On the other hand, inhibition of lipase in muscle may slow down clearance of circulating triacylglycerols. BOI extracts increased the fecal lipid content, possibly by inhibiting PL and other gastro-intestinal lipases, decreasing the digestibility of dietary fat. The accumulation of triglycerdes in the liver, induced by a high fat diet was reduced by the consumption of herbal extracts, possibly because the inhibition of gastric lipases and the subsequent reduction of the intestinal fat absorption and the reduction of the lipolysis. Such effects should, in turn, result in the suppression of hydrolysis and absorption of triglycerides. Since herbal extracts inhibited both lipase and amylase, reduced triglycerides in liver and lipolysis, the compounds in herbal extracts would have potential to reduce the body fat in animals. Though serum and liver TC and TG were increased in MC, the BOI extract treatment decreased ($p<0.05$) those values. Conversely, higher ($p<0.05$) fecal cholesterol outputs were measured in animals treated with herbal extracts as compared to MC. The liver Monaldehyde content was used to represent the liver peroxidation status, while liver GSH, CAT and SOD were used to evaluate the liver antioxidant capacity in the present study. Lower ($p <0.05$) liver MDA and higher ($p < 0.05$)

Table 2. the data of liver profile

	TC (µmol/gm)	TG (µmol/gm)	VLDL (µmol/gm)	HDL (µmol/gm)	HDL+VLDL (µmol/gm)	HDL/TC (µmol/gm)	SGOT (IU/L)	SGPT (IU/L)	SOD (U/mg of protein)	GSH (mg/g of tissue)
Normal control	7.40 ± 1.5**	18.36 ± 1.69**	3.67 ± 0.34**	0.94 ± 0.109**	8.96 ± 0.82**	0.14 ± 0.04	100.90 ± 14.53**	121.91 ± 6.31**	3.48 ± 0.02	4.21 ± 0.02
model control	20.32 ± 1.13	45.70 ± 1.3	9.14 ± 0.27	1.81 ± 0.12	21.79 ± 0.71	0.09 ± 0.01	302.34 ± 20.72	310.59 ± 20.47	1.59 ± 0.013	1.7 ± 0.078
BOI 50	18.438 ± 0.883	41.199 ± 1.187	8.24 ± 0.238	1.818 ± 0.226	19.821 ± 0.362	0.098 ± 0.007	242.322 ± 35.925	281.708 ± 45.230	1.94 ± 0.008	2.033 ± 0.225
BOI 100	17.712 ± 0.768	39.757 ± 0.639*	7.951 ± 0.128*	2.006 ± 0.063	19.371 ± 0.306*	0.113 ± 0.003	226.567 ± 12.704	232.944 ± 13.690	2.107 ± 0.002	2.067 ± 0.235
STD 10	10.01 ± 0.50**	39.10 ± 0.72**	7.82 ± 0.14**	2.57 ± 0.27**	19.63 ± 0.58	0.25 ± 0.03**	147.04 ± 13.16**	156.79 ± 7.5**	3 ± 0.079	3.89 ± 0.021
	Total glucose (µmol/gm)	**Amylase (IU/gm)**	**% inhibition**	**Lipase (IU/gm)**	**lipase inhibition**	**Catalase (µmol/gm)**	**% catalase inhibition**	**index of HMGR**	**% of MDA**	**% inhibition**
Normal control	108.68 ± 4.58**	0.25 ± 0.06**	7.84 ± 25.11**	2 ± 0.38	0 ± 19.24	1.91 ± 0.29**	–0.23 ± 15.03 **	1.80 ± 0.11**	20.39 ± 1.121**	
model control	248.90 ± 3.95	0.27 ± 0.007	-0.05 ± 2.63	2 ± 0.38	0 ± 19.24	0.18 ± 0.02	87.48 ± 1.02	2.03 ± 0.04	53.11 ± 3.85	–0.008 ± 7.25
BOI 50	187.457 ± 6.8**	0.094 ± 0.019	65.771 ± 6.966	1.556 ± 0.222	22.222 ± 11.111	1.018 ± 0.028*	45.263 ± 1.404*	2.804 ± 0.112	44.216 ± 2.926	16.746 ± 5.510
BOI 100	179.162 ± 1.333**	0.087 ± 0.013	68.404 ± 4.561	1.333 ± 0.385	33.333 ± 19.245	1.145 ± 0.023**	38.83 ± 1.187**	3.138 ± 0.166	42.574 ± 1.306	19.839 ± 2.459
STD 10	139.53 ± 3.66**	0.02 ± 0.00**	89.46 ± 2.63**	0.66 ± 0.38	66.66 ± 19.24	1.38 ± 0.03**	26.55 ± 1.77**	3.54 ± 0.09	36.68 ± 5.46**	30.92 ± 10.28**

Values are in mean ± SEM; Number of animals in each group =6; **P<0.01& *P<0.05 Vs Normal Control; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD 10 = treated with Atorvastatin (10mg/Kg).

GSH, CAT and SOD were observed in the NC and BOI extract treated groups than the others. In high fat diet fed groups, cholesterol synthesis was increased and the index of HMG-CoA/mevalonate ratio was decreased. In BOI and standard treated groups, cholesterol biosynthesis in liver was decreased while the index of HMG-CoA/mevalonate ratio was increased as compared to the MC group.

Histopathological studies for adipose tissue

Adipose tissues used were collected from subcutaneous region of the abdomen of rat. The histological appearance of epididymal adipocyte was irregular (exhibited heterogeneous sizes) in MC group compared to NC group. However, this morphological change mildly appears in BOI treated groups. The size of epididymal adipocytes was significantly bigger in MC group compared to other groups and the herbal treated groups showed moderate adipocyte size to that of NC group. These results suggest that BOI treated extract supplementation can inhibit lipid accumulation in epididymal adiypocyte tissue. No significant change in number of nuclei was observed when compared with Model control. Figure 1, expressed the histopathological studies on adipose tissue.

Histopathological studies for Liver

Fatty liver disease is a new clinic-pathological entity of emerging importance, now recognized as the most common cause of abnormal liver. It is characterized by a wide spectrum of liver damage, i.e. simple steatosis may progress to advanced fibrosis and to cryptogenic cirrhosis via steatohepatitis, and ultimately to hepatocellular carcinoma. Normal control and standard treated group liver tissues are of normal size. Observation of tissues in MC rats also showed large vaccules; fat degeneration; cumulative fatty cyst; vascular congestion moderate; dilatation marked; increase hepatocyte size; distinct enlargement of sinusoids; and sinusoidal dilatation with congestion. The livers of model control were clearly steatotic. In BOI treated group liver, few small fatty droplets, periportal inflammation, mild congestion; increased size of nucleus with prominent nucleolus; fibrosis were observed. Standard showed very few lipid droplets and the architecture of hepatocytes are found to be very similar to that of Normal control group. All treated livers have significantly reduced fat liver depots than the model control, as evaluated by H&E staining. The liver of treated animals had shown decreased lipid droplets, but there is a slight change

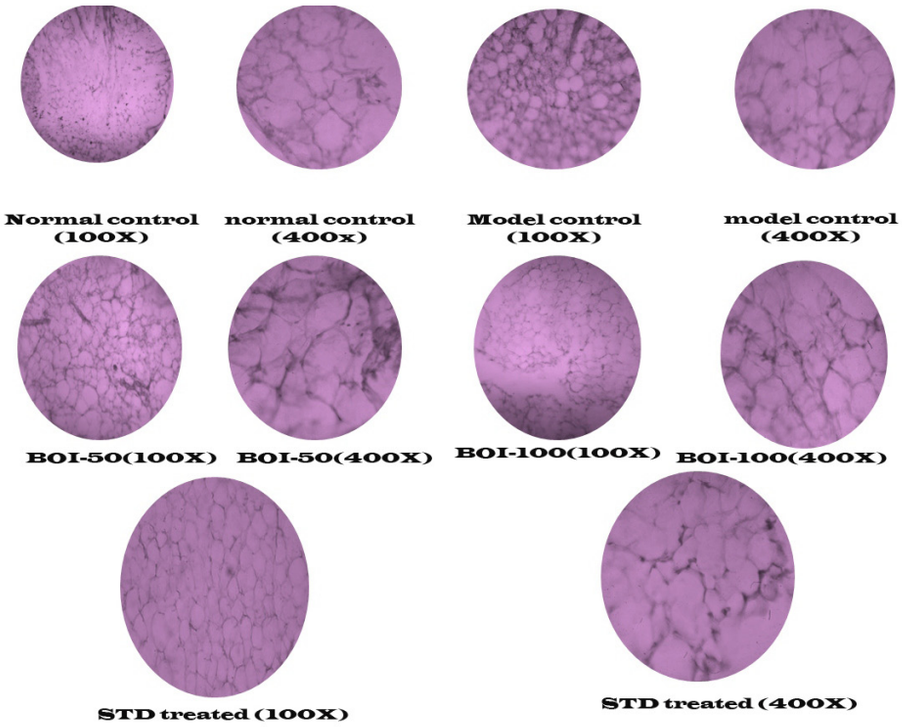

Figure 1. histopathological studies for adipose tissues (100X = hematoxylin–eosin stains with 100 magnification; 400X = hematoxylin–eosin stains with 400 magnification; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD treated = treated with Atorvastatin (10mg/Kg)).

in the morphology of hepatocytes. Figure 2 expressed the histopathological studies on liver.

Histopathological studies for Aorta

Normal control group and standard treated aortas were normal but in model control, atherosclerotic plaque was observed on aorta wall. Histopathology of aorta of MC rats indicated lesion with abnormal overlaying endothelium. Observation of tissues in MC rats also showed cholesterol deposits and fatty infiltration. BOI treated group aortas were similar to Normal control except with few inflammatory cells in the vessel wall and congestion. Figure 3 expressed the histopathological studies on aorta.

Histopathological studies for Heart muscle

Normal control group and standard treated groups were normal but in model control, few lipid droplets were present on wall. Histopathology of heart of MC rats indicated hyaline degeneration in muscles. BOI treated group aortas were similar to Normal control. Observation of tissues in herbal treated rats also showed mild hyaline degeneration in muscles. Figure 4 expressed the histopathological studies on heart.

Discussion

The oxidative modification of LDL and its accumulation in serum is a primary event in the proceeding of atherosclerosis. It is generally believed that antioxidants, which increase LDL oxidation resistance of the body, could inhibit atherosclerosis, though there is no direct evidence yet. The major advantages found in these pathological disorders have been in relation to fat deposition and serum triglycerides. In this way, a statistically significant decrease ($P<0.05$) has been found in the serum triglyceride levels of animals treated with BOI extracts as compared with MC. Some authors have associated this outcome with alterations in fat assimilation in the digestive tract and changes in triglyceride storage and mobilization in adipose tissue. Moreover, it has been reported that β-3-adrenergic agonists produce an increase in the rate of lipolysis and a decrease in lipid synthesis in adipose tissue. The reduction in lipase activity in serum and liver of BOI treated groups may be explained by these findings. The results suggested that BOI extracts reduced the extent of hypercholesteremia. It may be due to the inhibition of intestinal absorption of cholesterol and the acceleration of catabolism of cholesterol to bile acid. Also

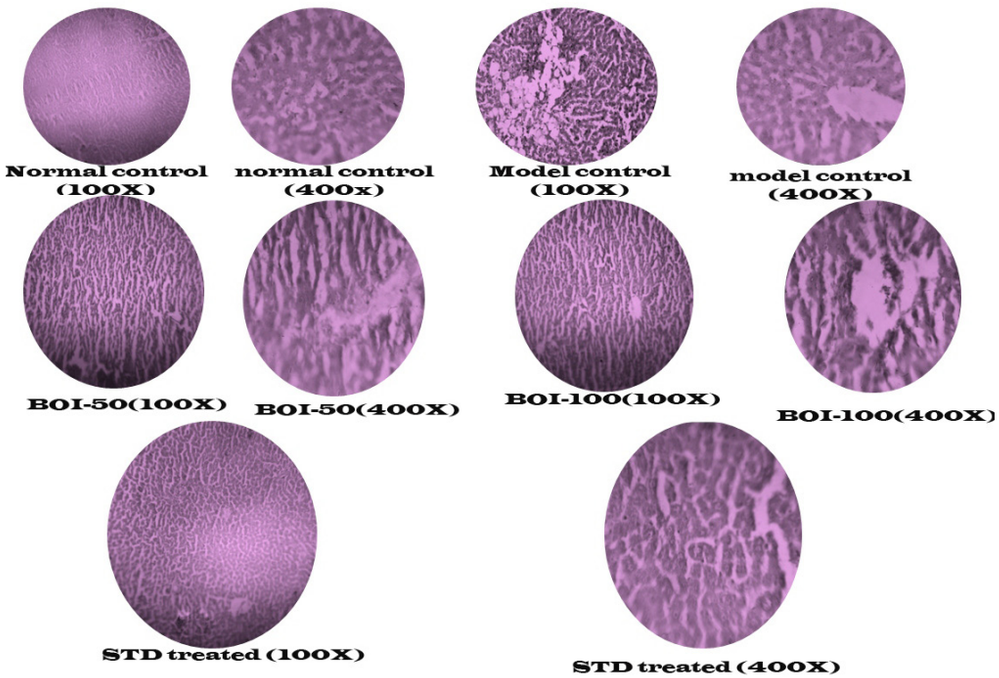

Figure 2. histopathological studies for liver (100X = hematoxylin–eosin stains with 100 magnification; 400X = hematoxylin–eosin stains with 400 magnification; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD treated = treated with Atorvastatin (10mg/Kg)).

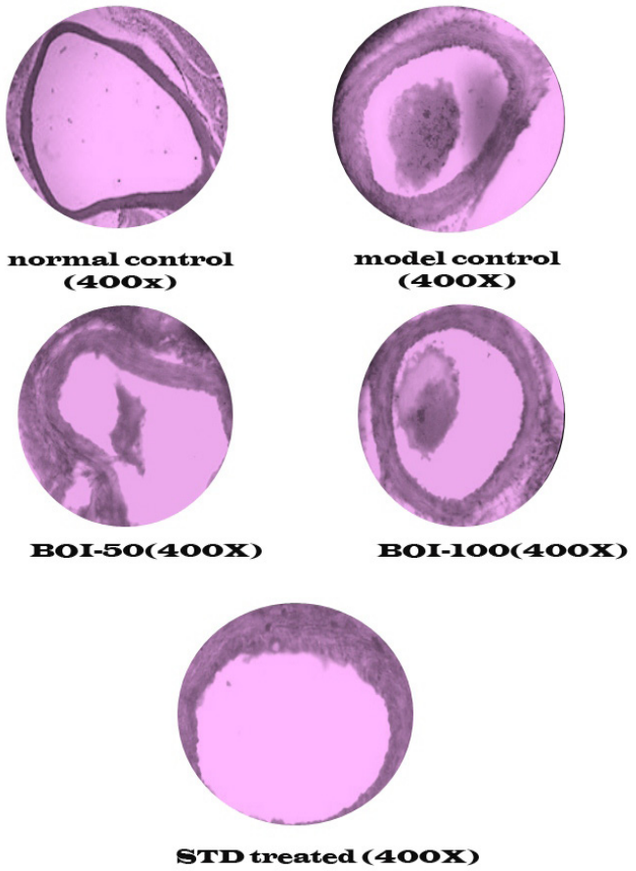

Figure 3. histopathological studies for aorta (100X = hematoxylin–eosin stains with 100 magnification; 400X = hematoxylin–eosin stains with 400 magnification; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD treated = treated with Atorvastatin (10mg/Kg)).

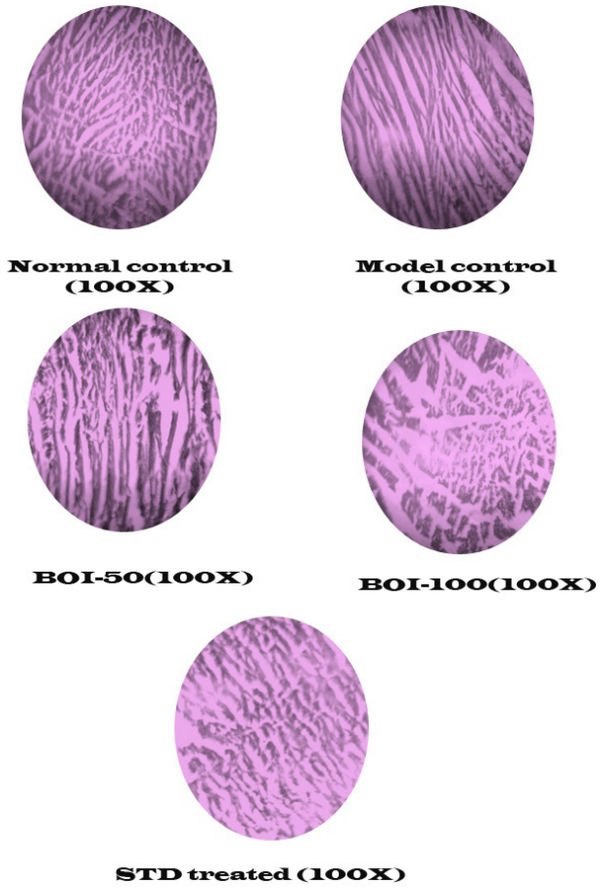

Figure 4. histopathological studies for heart (100X = hematoxylin–eosin stains with 100 magnification; 400X = hematoxylin–eosin stains with 400 magnification; BOI 50 = treated with 50mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; BOI 100 = treated with 100mg/kg dose of methanolic extract of *Boswellia ovalifoliolata* Bal. Henry; STD treated = treated with Atorvastatin (10mg/Kg)).

many phenolic compounds have been shown to possess hypolipidemic and antihypercholesterolemic activity by increasing the fecal cholesterol excretions and LDL receptor activity.[18,19] Atorvastatin which was used as standard drug in this study is a HMGR inhibitor which catalyzes the committed step in cholesterol biosynthesis. Statins are HMGR inhibitors that effectively lower serum cholesterol levels and are widely prescribed in the treatment of hypercholesterolemia. Rats treated with Atorvastatin showed marked reduction in all serum lipoproteins and increase in HDL level as compared with HFD group.[20] The inferred results were correlated with our previous reports that the phenolic compounds were the major contributors for antihypercholesterolemic activity of the extract. The present study suggests that both doses of BOI extracts are capable of exerting antihypercholesterolemic effects in high fat diet induced rats.

CONCLUSION

It can be concluded that the present study supports the folk information regarding the anti hypercholesterimic activity of *Boswellia ovalifoliolata* Bal. Henry by administering the macerated methanol extract of the plant to the rats which reduced hyperlipidemia. Further studies are required to isolate active principles from the extract to specify the extent of activity of each one to assess its antihypercholesterolemic effect.

ACKNOWLEDGEMENT

Authors acknowledge the management, Aditya institute of Pharmaceutical Sciences and Research and Nalanda College of Pharmacy for their technical support.

Authors acknowledge Mr. Madhavachetty, Botanist, S.V. University, Thirupati, Andhra Pradesh who was identified and authenticated the plant.

CONFLICTS OF INTEREST

We didn't receive any type of funds from government institution or any other funding agency or our college management. This work has done by our own expenditure.

REFERENCES

1. Verlecar XN, Jena KB, Chainy GBN. Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chemico-Biological Interactions. 2007;167: 219– 26.
2. Kromhout D. Epidemiology of cardiovascular diseases in Europe. Public Health Nutr. 2001; 4(2B): 441–57.
3. George AM, Demosthenes BP, Christos P, Dimitra X, George P, Christodoulos S. Prevalence of self-reported hypercholesterolemia and its relation to dietary habits, in Greek adults; a national nutrition and health survey. Lipids in Health and Disease. 2006; 5: 361–64.
4. Saravanan R, Rajendra Prasad N, Pugalandi KV. Effect of Piper betle leaf extract on alcoholic toxicity in the rat brain. J. Med. Food. 2003; 6: 261–65.
5. Kaesancini AY, Krauss RM. Cardiovascular disease and hyperlipidimemia. Cur. Opinion in lipidology. 1994; 5: 249–51.
6. Davey Smith G. Cholesterol lowering and mortality: the importance of considering initial level of risk. Int .Med. J. 1993; 306: 1367–73.
7. Davey Smith G, Pekkanen J. Should there be a moratorium on the use of cholesterol lowering drugs? Br. Med J. 1992; 304: 43–40.
8. Brown SL. Lowered serum cholesterol and low mood. Br. Med. J. 1996; 313: 637–38.
9. Speight TM. Drug treatment Principles and Practice of clinical Pharmacology and therapeutics. 3rd ed, Auckland: ADIS press Ltd. 1987; pp. 599.
10. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free. Radic. Biol. Med. 1996; 20: 707–27.
11. Suzuki T, Suzuki Y. Current topics of lipid dynamics and pathobiology in membrane lipid rafts. Biol .Pharm. Bull. 2006; 29(8): 1538–41.
12. Ryan D H. Diet and exercise in the prevention of diabetes. Int. J. Clin. Pract. 2003; 134: 28–35.
13. Sathis Kumar D, Srisutherson N, Pradeep Kumar Reddy B, Vinitha S, Yadhagiri Rao T, David Banji. Pharmacognostical study on *Boswellia ovalifoliolata* Bal. Henry, Journal of pharmacy research. 2011; 4(5): 1374–75.
14. Mitchell RN, Cotran RS. In: Robinsons Basic Pathology. Edtn 7. Harcourt Pvt. Ltd, New Delhi, India. 2000; 33–42.
15. Singleton VL, Orthofer R, Lamuela Raventos RM. Analysis of total phenols & other oxidation substrates & antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 1999; 299: 152–78.
16. Sakuntala Devi PR, Adilaxmamma K, Srinivasa Rao G, Srilatha Ch, Alpha Raj M, Safety evaluation of alcoholic extract of *Boswellia ovalifoliolata* stem-bark in rats, Toxicological international. 2012; 19(2): 115–20.
17. Vishnu Kumar, Pradyumn singh, Ramesh Chander, Farzana Mahdi, Sushma Singh, Ranjana Singh, Ashok kumar Khanna et al. Hypolipidemic activity of Hibiscus rosa sinensis root in rats. Indian journal of biochemistry and biophysics. 2009; 46: 507–10.
18. Chan PT, Fong WP, Cheng YL, Huang Y, Chen ZY. Jasmine green tea epicatechins are hypolipidemic in hasters fed a high fat diet. Journal of Nutrition. 1999; 129: 1094–101.
19. Tikkanen MJ, Wahaink OS, Vimha V, Adcercrentz H. Effect of Soyabean phytoestrogen intake on LDL oxidation resistance, Proc. Natl. Acad. Sci. 1998; 95: 3106–110.
20. Varsha D, Shubhangi S, Mangesh P, Naikwade NS. Antihyperlipidemic Activity of Cinnamomum tamala Nees on High Cholesterol Diet Induced Hyperlipidemia. International Journal of Pharm Tech Research, 2010; 2(4): 2517–21.

Qualitative and Quantitative Analysis of *Nyctanthes arbortristis* Linn leaf extracts by HPTLC

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Submission Date: 18-2-2014

Accepted Date: 24-3-2014

ABSTRACT

Nyctanthes arbortristis L. (Oleaceae) or "night jasmine" is cultivated for its fragrant flowers and is widely used in traditional systems of medicine as an anthelmintic, cholagogue, laxative and antimalarial. The plant has been studied pharmacognostically and certain standards are available. However there are no reports on HPTLC quality assessment of *Nyctanthes arbortristis*. Hence the present study involves the development of qualitative HPTLC fingerprint profile of n-hexane, ethyl acetate, methanol extracts of leaves of *N. arbortristis* followed by quantitation of marker compounds â-sitosterol, â-amyrin in n-hexane extract and caffeic acid in ethyl acetate extract. The developed methods were used for comparison of plant extracts with a few commercial formulations containing *N. arbortristis*. These HPTLC methods can be used easily for evaluation of quality of plants collected from different sources as well as for commercial formulations containing *N. arbortristis*.

Keywords: *Nyctanthes arbortristis,* commercial formulations*,* HPTLC qualitative and quantitative evaluation

INTRODUCTION

Nyctanthes arbortristis (Family: Oleaceae) commonly known as night jasmine or coral Jasmine (English) or Harsingh-har (Hindi) is a native of India and is widely distributed throughout India, Srilanka, Bangladesh and other parts of South East Asia.[1–4] It is cultivated all over the world for its fragrant flowers.[2] The bright orange corolla tubes of the flowers contain saffron - yellow colouring matter, this was formerly used for dyeing silk.[2,5,6]

Traditionally the plant was used by tribals (Santals) in snake bite and bites of wild animals. It was also used in cachexia, cancer, sores, ulcers, dysentery, and menorrhagia and enlargement of the spleen.[7] The bark has been used for treatment of bronchitis. The bark in combination with Arjun bark (*Terminalia arjuna*) was considered to be useful in internal injuries, healing of wounds including fractured bones.[8] Expressed juice of the leaves act as cholagogue, laxative and mild bitter tonic. Flowers have a bitter bad taste and have been used as a stomachic, carminative, astringent to the bowels. The powdered seeds were used to cure piles and skin diseases.[1,2,9–13] The decoction of leaves of this plant is widely used in Ayurvedic medicine for the treatment of sciatica, arthritis, as well as tonic, laxative and cholagogue.[1] Ethnobotanical surveys show that the plant has been used for prevention of malaria.[14]

The plant has been studied pharmacognostically and pharmacologically. [15,16] Phytochemical investigation of the plant has shown presence of different phytoconstituents in different parts of *N. arbortristis* plant (Table 1). Various extracts of the plant have shown different pharmacological activities (Table 2). Certain analytical standards for the plant extracts/constituents are available. Nyctanthic acid has been considered as one of the major constituents and it is estimated by TLC densitometric method.[15] Total glycosides have been estimated using TLC method.[17] Total phenolics and flavonoids were

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DOI: 10.5530/pj.2014.3.17

Table 1. Chemical constituents isolated from different parts of *Nyctanthes arbortristis*

Plant part	Chemical constituent	Reference
Bark	• A glycoside, • Two alkaloids one is soluble in water and other soluble in chloroform • Flavone glycoside	[2] [19]
Leaves	• Terpenoids Nyctanthic acid, Lupeol, Oleanolic acid, βamyrin, β-Sitosterol. • Phenolic compounds and flavanol glycosides Astragelin (kaempferol-3-glucoside), Nicotiflorin (kaempferol-3-rhamnoglucoside); Caffeic acid. • Iridoid glycosides 6β- hydroxyl loganin, Arborsides A, B, C and D, nyctanthoside Benzoic esters of loganin - 6,7-di- O- benzoylnyctanthoside, 6-O-trans-cinnamoyl- 6β- hydroxyl loganin, 7-O-trans-cinnamoyl-6β- hydroxyl loganin, Desrhamnosyl verbascoside • Carbohydrates D-mannitol, glucose, fructose. • N-alkanes Hentriacontane, tritriacontane	[20–22] [24;26–28] [29]
Flowers	• Oil This contains α pinene, p-cymene, 1-hexanol, methyl heptane, Phenyl acetaldehyde, 1-decanol and anisaldehyde. • Glycosides 6-O-trans cinnamoyl 7-O acetyl 6β- hydroxyl loganin, 6β- hydroxyl loganin , Arborside C and Nyctanthoside • Crocetin esters β -monogentiobioside ester of and β-digentiobioside ester of α-crocetin. • Benzofuranone derivatives 3, 3a, 7, 7a-tetra hydro-3a-hydroxy-6(2H)-benzofuranone • A carotenoid aglycone-crocetin	[30] [31] [32] [33] [34]
Seeds	• Iridoid glycosides Arbortristosides A, B, D, E. • Glycerides,Lignoceric acid, Stearic acid, Palmitic acid, myristic acids	[23; 25; 27] [35]

determined by spectrophotometrically.[17,18] However there were no reports on HPTLC profile of *N. arbortristis*. Hence the present study involves development of qualitative HPTLC fingerprint profile of n hexane, ethyl acetate, methanol extracts of leaves of *N. arbortristis* Linn along with HPTLC method for quantification of marker compounds β-sitosterol, β-amyrin in n-hexane extract and caffeic acid in ethyl acetate extract. These methods form the basis of standardization of commercial formulations containing *N. arbortristis*

MATERIALS AND METHODS

Plant Material

Leaves of *Nyctanthes arbortristis* were collected from Punjabi University campus (Patiala, India) during the months of February and March, 2007. The leaves were authenticated by Dr. H. B. Singh, Head, Raw Materials Herbarium & Museum, National Institute of Science Communication and Information Resources (CSIR), New Delhi, 110067. A voucher specimen number: NISCAIR/RHMD/consult/2007-08/870/54 has been deposited in the same herbarium.

Chemicals

All chemicals used were of analytical grade and were purchased from S.D. Fine Chemicals Limited, Mumbai.

Refernce Standards

β-Sitosterol, β-amyrin and caffeic acid were gifted by National Institute of Pharmaceutical Education and Research (NIPER, Mohali).

Commercial Formulations

Three commercial formulations (Table 3) were procured from local market.

HPTLC equipment

A Camag TLC/HPTLC Integration (CATS V_4.04; S/N 0511A011); TLC Scanner 3 (V_1.14); Camag Linomat IV sample spotter; Camag glass twin trough chamber (20 cm × 10 cm)

Precoated Silica gel 60 TLC plate (0.2 mm thickness, 10 × 10 cm) (E. Merck); Temperature 25 ± 2°C, Relative humidity 40%

Table 2. Pharmacological activities reported from *Nyctanthes arbortristis*

Plant part	Extract/constituent	Activity observed	References
Leaves	Water soluble portion of the alcoholic extract	• Anti-inflammatory activity • Analgesic, antipyretic and ulcerogenic activities. • Prevent TNF- α accumulation in arthiritic and in induced lung injury in mice • Tranquilizing, depression of spontaneous motor activity, hypothermic and purgative activity	[37] [38] [39–40] [41]
	Ethanolic extract	• Trypanocidal activity • Bronchodilatory	[42] [43]
	Aqueous extract	Stimulation of Ach E activity in immuno suppresed mice	[44]
	• Methanolic extract • Phenolics of methanol extract	Anixolytic, nootropic and anti stress activity Anti oxidant activity	[45] [46]
	Acetone soluble fraction of ethyl acetate extracts.	Anti oxidant activity	[18; 47]
	Polyphenols from petroleum ether extract	Anti oxidant activity	[48]
	Alcoholic and aqueous extracts	Hepatoprotective activity	[49]
	β-sitosterol from petroleum ether extract	Analgesic and anti-inflammatory activity.	[50]
	Crude extract and RPHPLC fractions	Antiplasmodial activity	[51]
	Ethanol extract	Antimalarial	[52]
Stem Bark	Methanol extract of stem bark	Anti spermatogenic activity	[53]
Bark	Flavone glycoside	Anti-inflammatory	[19]
Flowers	Ethanolic extract and isolated carotenoid from extract.	Anti-inflammatory	[54]
	Chloroform and ethyl acetate extracts and isolated compound (3, 3a, 7, 7a - tetra hydro-3a-hydroxy-6(2H)-Benzofuranone	Antibacterial activity	[33; 36]
	Chloroform extract and its isolated compound NCS-2	Anti filarial activity	[56]
	4-hydroxyhexahydrobenzofuran-7-one	Cytotoxic activity	[55]
	Hot infusion	Moderate dose dependent sedative activity in male rats	[57]
Seeds	Iridoid glycosides (arbortristosides A, B, C and 6β hydroxyl loganin)	*In vivo* and *in vitro* anti leishmanial activity	[25; 58; 59]
	50% ethanolic extracts	Immunomodulatory activity	[60]
	Arbortristosides A and C	Significant mast cell stabilizing activity	[15]
	Ethanolic extracts and isolated compounds arbortristoside A, B	Anti viral activity	[61]
	Arbortristoside A	Anti-inflammatory and antinociceptive activity	[62]
Different parts	$CHCl_3$ and ethyl acetate extract of leaf, fruits, flowers, and seeds	Antibacterial against gram negative bacteria	[17]
	Water-soluble portion of the ethanol extracts of flowers, barks, seeds and leaves	CNS depressant activity	[63]
	Ethanolic extract of seeds and leaves	Diuretic	[64]

Preparation of leaf extracts

Fresh leaves were dried under shade at room temperature and powdered by electrical grinder. Powdered drug (120 g) was subjected to successive Soxhlet extraction with n-hexane, ethyl acetate and methanol. The extracts were collected and evaporated by Rotatory vacuum evaporator. The extractive values were calculated with reference to air dried drug.

Preparation of extracts of commercial formulations

a) From poly herbal formulation (Deep act OS)

Five tablets were powdered and 1 g each was used for extraction with n-hexane, ethyl acetate and methanol separately, by maceration. The resultant extracts were evaporated to dryness using a water bath. The extractive

Table 3. Constituents of commercial formulations containing *Nyctanthes arbortristis*

S.No	Formulations	Constituents	Manufacturer
1	Deep act OS tablets	Each tablet contains extracts of Shallaki 150mg Guggulu 120mg Parijatha 43mg Haridra 40mg Haritaki 25mg Shunthi 15mg	Lupin Herbal (Mumbai)
2	Mother tincture (1)	Alcoholic 59% v/v extract of *N.arbortristis*	Ralson Remedies (p) Ltd (Delhi)
3	Mother tincture (2)	Alcoholic 59% v/v extract of *N. arbortristis*	Medi synth.pvt. Ltd (Navi Mumbai)

values were calculated with reference to the weight of drug taken.

b) From mother tinctures

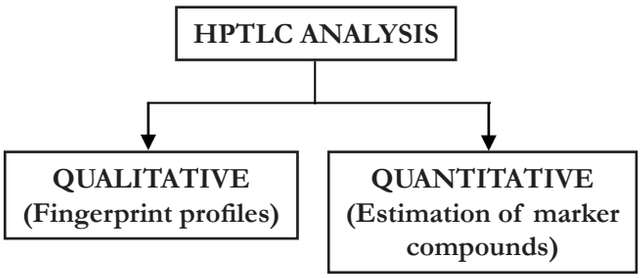
In the present study two mother tinctures of different companies were examined. Same extraction method was used for two mother tincture.

Mother tincture (50 ml) was evaporated to dryness by Rotavapour. The dried residue was subjected to successive fractionation by n-hexane, ethyl acetate and methanol by maceration. The extracts were evaporated to dryness by using a water bath. The extractive values were calculated with reference to the volume of the drug taken.

TLC profile of different extracts

The n-hexane, ethyl acetate and methanol extracts obtained from plant as well as commercial formulations were subjected to thin layer chromatographic (TLC) analysis. The solvent system of different extracts was given in the following (Table 4).

HPTLC Analysis



a) Qualitative analysis

Qualitative analysis was done by comparing Rf values, colour of the spot obtained, UV absorbance characters of the spots and fluorescent nature of the spots.

- n-hexane extracts

HPTLC profiles of n-hexane extracts of *N.arbotristis,* poly herbal formulation (Deep act OS) were developed and compared with standards β- sitosterol, β- amyrin.

- Ethyl acetate extracts

HPTLC profiles of ethyl acetate extracts of *N. arbotristis,* poly herbal formulation, mother tincture 1 and 2 were developed and compared with standard caffeic acid.

- Methanol extracts

HPTLC profile of methanol extracts of *N.arbotristis,* poly herbal formulation, mother tincture 1 and 2 were developed and compared with methanol extract of *N. arbotristis*.

Preparation of samples

From the dried residue of different extracts, the samples were prepared in the following way (Table 5).

Preparation of Standards

- β- Sitosterol - A stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of β- sitosterol in 10 ml of hexane.
- β- Amyrin - A stock solution of 0.1 mg/ml was prepared by dissolving 1 mg of β- amyrin in 10 ml of hexane.
- Caffeic acid - A stock solution of 0.01 mg/ml was prepared by dissolving 1mg of caffeic acid in 100 ml of ethyl acetate.

Solvent system

The following solvent systems, derivatizing agents, and scanning wavelengths were used for different extracts (Table 6).

Method development

- n-hexane extracts

The n hexane extracts of *N. arbortristis* (2 µl), poly herbal formulation (1 µl) were applied in triplicate on precoated

Table 4. Different solvent systems used in TLC for different extracts

Samples	Solvent system	Derivatizing agent
n-hexane extracts (plant, poly herbal formulation)	Toluene: EtOAc (8:2)	Anisaldehyde sulphuric acid.
Ethyl acetate extracts (plant, poly herbal formulation, mother tincture 1 and 2)	EtOAc: formic acid: glacial acetic acid: H_2O (10:11:11:27)	NP-PEG reagent and observed under UV 366nm
Methanol extracts (plant, poly herbal formulation, Mother tincture 1 and 2)	$CHCl_3$: MeOH (8:2)	Vanillin in sulphuric acid

Table 5. Preparation of samples for qualitative HPTLC analysis

Extracts	Plant		PHF		MT_1		MT_2	
	stock solution	sample volume	stock solution	sample volume	stock solution	sample volume	stock solution	sample volume
n hexane	20 mg/ml	2 µl	20 mg/ml	1 µl	–	–	–	–
EtOAc	10 mg/ml	4 µl	10 mg/ml	4 µl	5 mg/ml	3 µl	5 mg/ml	3 µl
MeOH	20 mg/ml	4 µl	10 mg/ml	4 µl	10 mg/ml	4 µl	10 mg/ml	4 µl

PHF- Poly herbal formulation, MT1- Mother tincture 1, MT2- Mother tincture 2.

Table 6. Solvent systems used in HPTLC for different extracts

Extracts	Solvent system	Derivatizing agents	Scanning wave length
n-hexane	Toluene: EtOAc (8:2)	Anisaldehyde sulphuric acid	560 nm
EtOAc	Toluene: EtOAc: Acetic acid (5:4:1)	NP-PEG reagent	366 nm
MeOH	EtOAc: MeOH (8:2)	Anisaldehyde sulphuric acid	560 nm

EtOAc- Ethyl acetate, MeOH- Methanol, NP-PEG – Natural product- Poly ethylene glycol reagent.

silica gel TLC plate using linomat IV sample spotter. β-sitosterol (2 µl), β-amyrin (2 µl) were also applied on the same plate. The plates were developed in the solvent system mentioned in table 5 in glass twin trough chamber. The plates were developed up to 8cm. After development the plates were dried in air. Then the plates were derivatized with anisaldehyde sulphuric acid by dipping method. The plates were heated at 100ºC for 5 to 10 minutes. The plates were scanned at the wavelength of 560 nm.

- Ethyl acetate extracts

The ethyl acetate extracts of *N. arbortristis* (4 µl), poly herbal formulation (4 µl), mother tincture 1 and 2 (3 µl) were applied in triplicate on precoated silica gel TLC plate using linomat IV sample spotter. Caffeic acid (2 µl) was also applied on the same plate. The plates were developed in the solvent system mentioned in table 10 in glass twin trough chamber. The plates were developed up to 8cm. After development the plates were dried in air. Then the plates were derivatized with NP-PEG reagent by dipping method. The plates were scanned at the wavelength of 366 nm.

- Methanol extracts

The methanol extracts of *N. arbortristis* (4 µl), poly herbal formulation (4 µl), mother tincture 1 and 2 (4 µl) were applied in triplicate on precoated silica gel TLC plate using linomat IV sample spotter. The plates were developed in the solvent system mentioned in table 10 in glass twin trough chamber. The plates were developed up to 8 cm. After development the plates were dried in air. Then the plates were derivatized with anisaldehyde sulphuric acid reagent by dipping method. The plates were heated at 100ºC for 5 to 10 minutes. The plates were scanned at the wavelength of 560 nm.

b) Quantitative analysis

This involves quantitation of constituents by using calibration curves of standards. Amount or concentration was calculated by comparing peak area/peak height of sample with peak area/peak height of standard.

Development of calibration curves

- β-sitosterol (stock solution 0.1 mg/ml)

Aliquots of stock solution - 3 µl, 5 µl, 7 µl, 9 µl, 11 µl, 13 µl corresponding to 0.3 µg, 0.5 µg, 0.7 µg, 0.9 µg, 1.1 µg, 1.3 µg of β-sitosterol were applied on precoated silica gel 60 TLC plate. The plate was developed in solvent system (Toluene: ethyl acetate, 8:2) in glass twin trough chamber to a distance of 8 cm. After development the plate was dried in air. Then the plate was derivatized with

anisaldehyde sulphuric acid reagent by dipping method. The plate was heated at 100ºC for 5 to 10 minutes. The plate was scanned at the wavelength of 560 nm. The peak areas were recorded. The calibration curve of β-sitosterol was prepared by plotting peak areas versus concentration of β-sitosterol applied.

- β-amyrin (stock solution 0.1 mg/ml)

Aliquots of stock solution - 1 µl, 3 µl, 5 µl, 7 µl, 9 µl, corresponding to 0.1 µg, 0.3 µg, 0.5 µg, 0.7 µg, 0.9 µg of β-amyrin were applied on precoated silica gel 60 TLC plate. The plate was developed, derivatized, scanned as similar as β-sitosterol. The peak areas were recorded. The calibration curve of β-amyrin was prepared by plotting peak areas versus concentration of β-amyrin applied.

- Caffeic acid (stock solution 0.01 mg/ml)

Aliquots of stock solution (10 µl × 4 times), (20 µl × 2 times), corresponding to 0.1 µg, 0.2 µg of caffeic acid were applied on precoated silica gel 60 TLC plate. The plate was developed in solvent system (Toluene: EtOAc: acetic acid, 5:4:1) in glass twin trough chamber to a distance of 8 cm. After development the plate was dried in air. The plate was derivatized with NP-PEG reagent by dipping method. Then the plate was scanned by UV 366 nm. The peak areas were recorded. The calibration curve of caffeic acid was prepared by plotting peak areas versus concentration of caffeic acid applied.

Quantitation of β-sitosterol, β-amyrin in n-hexane extracts

β-sitosterol and β-amyrin was quantified in n-hexane extracts of *N. arbortristis* and poly herbal formulations. The plates were developed, derivatized and scanned as similar as qualitative analysis of n-hexane extracts. The peak areas were recorded. The concentration of β- sitosterol and β-amyrin were calculated by comparing peak areas with calibration curves of both.

Quantitation of caffeic acid in ethyl acetate extracts

Caffeic acid was quantified in ethyl acetate extracts of *N.arbortristis,* poly herbal formulation, mother tincture 1 and 2. The plates were developed, derivatized and scanned as similar as qualitative analysis of ethyl acetate extracts. The peak areas were recorded. The concentration of caffeic acid was calculated by comparing peak areas with calibration curve of caffeic acid.

RESULTS AND DISCUSSION

Procurement of leaves and preparation of extracts

Leaves of *N. arbortristis* were collected from Punjabi University campus and authenticated by NISCAIR, New Delhi, India as well as by critical morphological and microscopical examination and by comparison with standards available.[15] Commercial formulations, Deep Act OS (Lupin herbal), mother tinctures of *Nyctanthes arbortristis* from two different companies (Ralson Remedies, Medi Synth ltd) were purchased and used for our study. Three extracts (n hexane, ethyl acetate, and methanol) of each were prepared from the leaves of *N. arbortristis* and commercial formulations in order to separate the phytoconstituents on the basis of polarity. The n-hexane extracts are reported to contain β- sitosterol, β- amyrin.[20] The ethyl acetate extracts are reported to contain caffeic acid.[65]

Commercially available poly herbal formulation (Deep act OS) and mother tinctures containing *N. arbortristis* were processed. Sample preparation of both was designed in such a way so as to extract phytoconstituents, differentiated on the basis of polarity similar to the preparation of extracts from the plant material.

The percentage yields of different extracts of plant and commercial formulations were showed in (Table 7).

Thin layer chromatographic profiles

Results of thin layer chromatographic studies of different leaf extracts are reported in (Table 8).

Qualitative HPTLC analysis

Qualitative HPTLC examination revealed the presence of the marker compounds (β- sitosterol and β-amyrin) in n- hexane extract of plant and poly herbal formulation; caffeic acid in ethyl acetate extract of plant, poly herbal formulation, mother tincture 1 and 2 while in the methanol extracts, peak at Rf 0.34 was present in plant, mother tincture 1 and 2 and absent in poly herbal formulation. Peak at Rf 0.43 was present in plant, poly herbal formulation, mother tincture 1 and 2. This was done to confirm the presence of known marker compounds in plant material and commercial formulations.

HPTLC fingerprint profile of n-hexane extracts

The qualitative fingerprint profile of n-hexane extracts has been described using well knowns table compounds β-sitosterol, β-amyrin as marker compounds. The standards

Table 7. Percentage yields of extracts of plant and commercial formulations

S.No	Sample	n-hexane	Ethyl acetate	Methanol
1	*Nyctanthes arbortristis*	1.6% w/w	4.5% w/w	4.0% w/w
2	Poly herbal formulation (Deep act OS)	2.5% w/w	4.5% w/w	3.0% w/w
3	Mother tincture (1)	–	2.5% w/v	1.5% w/v
4	Mother tincture (2)	–	1.6% w/v	1.5% w/v

Table 8. TLC results of different extracts of leaves of *N. arbortristis* and its commercial formulations

Extract	Solvents with ratio	No. of spots	Rf Values
n-hexane extract of Plant PHF	Toluene:ethyl acetate, 8:2	3	0.52, 0.73, 0.78
		3	0.52, 0.69, 0.85
Ethyl acetate of Plant PHF MT_1 MT_2	Ethyl acetate:formic acid:glacial acetic acid:water,100:11:11:27	1	0.90
		1	0.90
		1	0.90
		1	0.90
Methanol extract of Plant PHF MT_1 MT_2	Chloroform:methanol,8:2	2	0.86, 0.87
		1	0.93
		2	0.78, 0.86
		2	0.56, 0.61

PHF- Poly herbal formulation, MT1- Mother tincture 1, MT2- Mother tincture 2.

β-sitosterol, β- amyrin were showed a purple coloured peak with the Rf Value 0.41, 0.54 respectively. The result of HPTLC analysis of n-hexane extract of the plant is detailed in plate 1, (Table 9).

The HPTLC profile of n-hexane extracts of *N. arbortristis* showed peak number 3, 5 at Rf value 0.40, 0.53 which correspond to β-sitosterol, β- amyrin respectively, thus showing presence of these well known compounds in the n- hexane extract of plant.

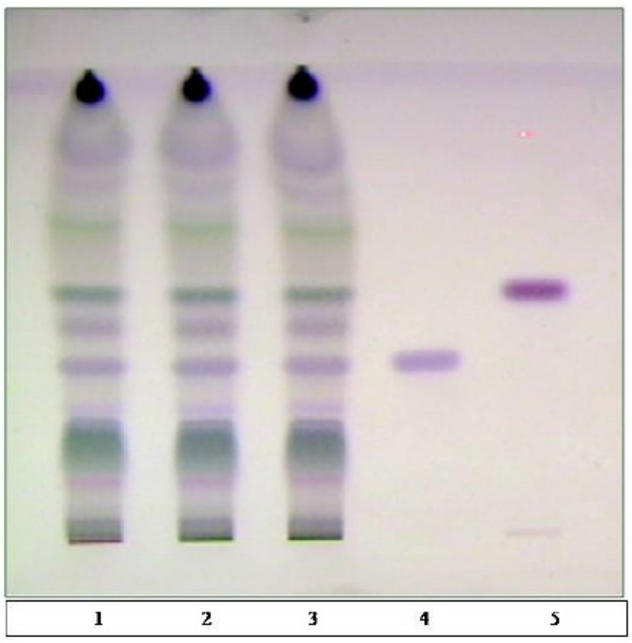
The following result was obtained with poly herbal formulation containing *N.arbortristis* (Plate 2, (Table 10).

The HPTLC profile of n-hexane extract of poly herbal formulation (Deep act OS) showed peak No 5, 7 at Rf value 0.40, 0.54 which correspond to β-sitosterol, β- amyrin respectively.

HPTLC fingerprint profile of ethyl acetate extracts

The qualitative fingerprint profile of ethyl acetate extracts has been described using caffeic acid as marker compound. The standard caffeic acid was showed light blue fluorescent peak with Rf value 0.53. The result of HPTLC analysis of ethyl acetate extract of plant is detailed in plate 3, (Table 11).

The HPTLC profile of ethyl acetate extracts of *N. arbortristis* showed peak No 2 at Rf value 0.52 and this corresponds to caffeic acid.The following results were obtained with commercial formulations containing *N. arbortristis* (Plates 4-5),(Table 12).

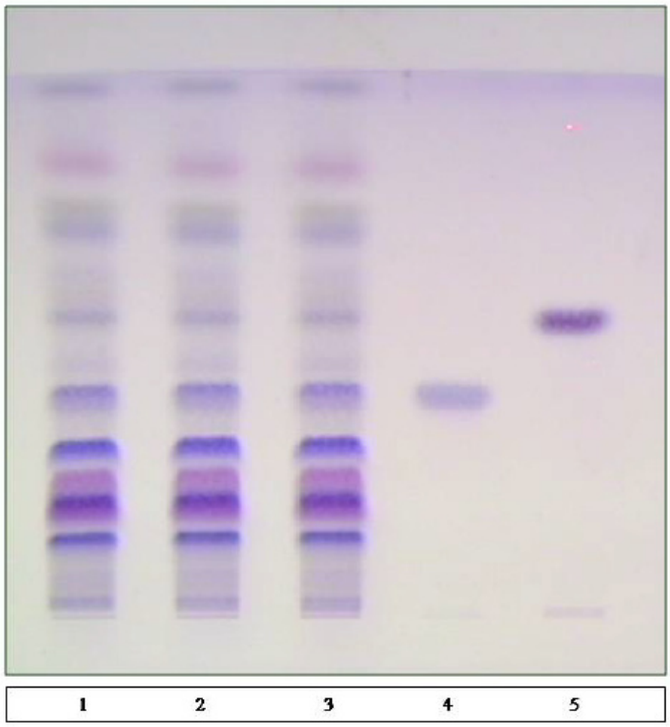


(1 - n hexane extract of plant; 2 - n -hexane extract of plant; 3 - n -hexane extract of plant; 4 - β sitosterol; 5 - β amyrin)

Plate 1. HPTLC profile of n hexane extract of plant and standards.

Table 9. HPTLC profile of n hexane extract of Plant and standards

Track No	Sample	Peaks	Rf value at max	Height of peak at max	AUC of peak
1,2,3	*Nycatanthes abortristis*	1	0.03	42.3	472.7
		2	0.20	217.9	16904.0
		3	0.40	97.1	3105.7
		4	0.43	87.8	2932.1
		5	0.53	141.8	3593.4
		6	0.64	34.9	1405.2
		7	0.72	17.7	439.8
		8	0.78	37.0	1477.7
4	β- sitosterol	1	0.40	162.9	6314.0
5	β- amyrin	1	0.54	313.5	10674.0

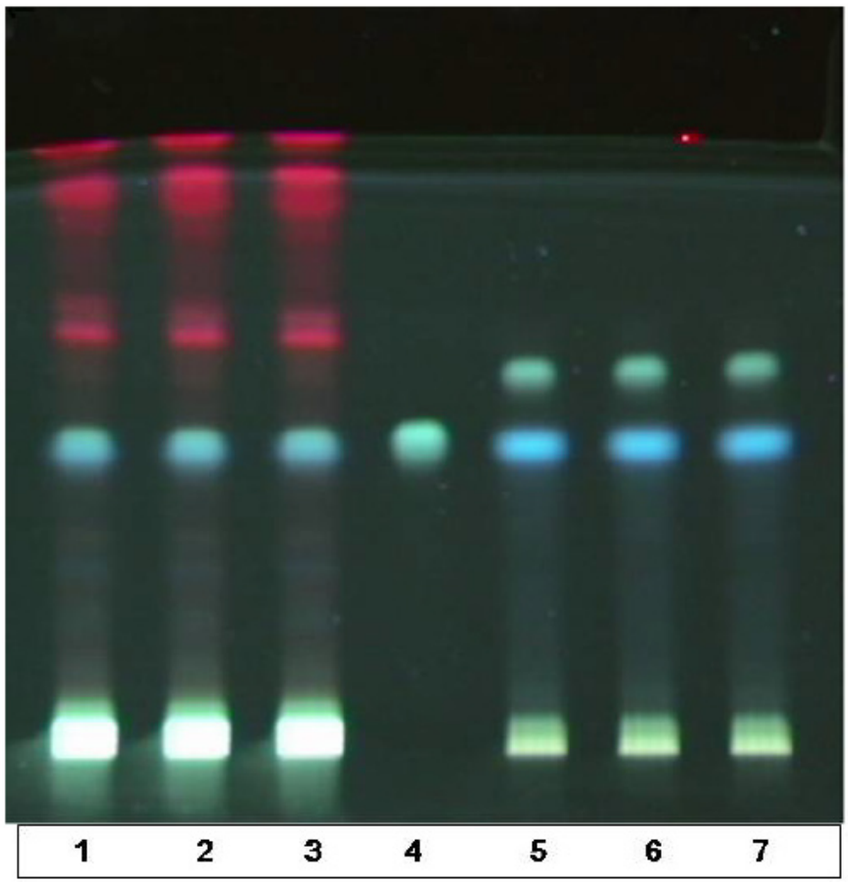


(1 - n hexane extract of PHF; 2 - n hexane extract of PHF; 3 - n hexane extract of PHF; 4 - β sitosterol; 5 - β -amyrin) (PHF - poly herbal formulation, Deep act OS)

Plate 2. HPTLC profile of n-hexane extract of PHF and standards.

Table 10. HPTLC profile of n hexane extracts of PHF and standards

Track No	Sample	Peaks	Rf value at max	Height of peak at max	AUC of peak
1,2,3	Poly herbal formulation (Deep act OS)	1	0.03	81.0	795.0
		2	0.14	265.5	7138.5
		3	0.20	240.3	13620.0
		4	0.31	289.3	7114.1
		5	0.41	172.6	5108.3
		6	0.46	24.3	466.9
		7	0.54	95.3	3290.3
		8	0.62	30.8	554.1
		9	0.71	100.6	4692.1
		10	0.83	80.7	2865.9
4	β-sitosterol	1	0.40	166.0	6557.6
5	β- amyrin	1	0.54	331.3	10939.5



(1 - EtOAc extract of plant; 2 - EtOAc extract of plant; 3 - EtOAc extract of plant; 4 - Caffeic acid; 5 - EtOAc extract of MT_1; 6 - EtOAc extract of MT_1; 7 - EtOAc extract of MT_1) (EtOAc – ethyl acetate; MT - mother tincture)

Plate 3. HPTLC of ethyl acetate extract of plant, Mother $Tincture_1$ and standard.

Table 11. HPTLC profile ethyl acetate of extract of plant and standard

Track No	Sample	Peaks	Rf value at max	Height of peak at max	AUC of peak
1,2,3	*Nyctanthes arbortristis*	1	0.09	11.9	84.5
		2	0.52*	44.6	1858.5
		3	0.68	71.6	3052.8
4	Caffeic acid	1	0.52	93.1	4468.2

* Blue fluorescent spot.

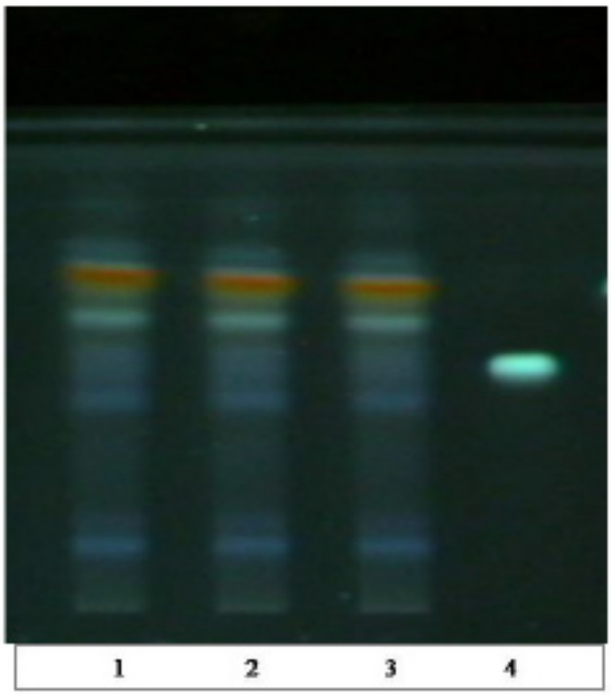
HPTLC fingerprint profile of methanol extracts

The HPTLC fingerprint profile of methanol extracts of commercial formulations have been described and compared with methanol extract of *N. arbortristis*. Here no external standard was used. The following result was obtained with methanol extract of plant and commercial formulations of plant (Plate 6), (Table 13).

From the results mentioned above it was observed that some related peaks at Rf value 0.34, 0.45 were present in the HPTLC profile of all four extracts. The colour of resolved bands in all four extracts was same under visible region. These may be due to the presence of same compounds, but the compounds cannot be specified in the absence of any standards.

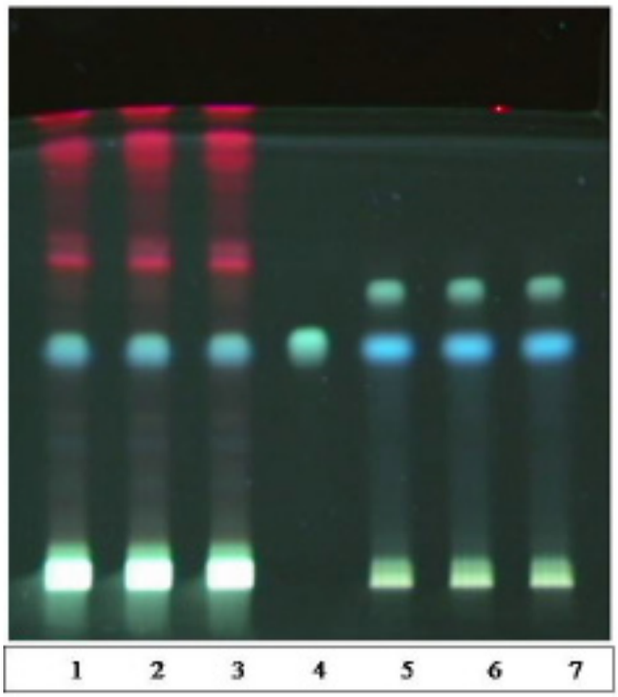
Quantitative HPTLC analysis

Although the presence of marker compounds (β- sitosterol, β-amyrin and caffeic acid) have been reported in the plant,[15,20,65] quantitative estimation had not been carried out to determine the amount of the well known marker compounds in the plant extracts. In the present study



(1 - EtOAc extract of PHF; 2 - EtOAc extract of PHF; 3 - EtOAc extract of PHF; 4 - Caffeic acid) (EtOAc – ethyl acetate; PHF – poly herbal formulation)

Plate 4. HPTLC profile ethyl acetate extract of Poly Herbal Formulation and standard.



(1 - EtOAc extract of plant; 2 - EtOAc extract of plant; 3 - EtOAc extract of plant; 4 - Caffeic acid; 5 - EtOAc extract of MT_2; 6 - EtOAc extract of MT_2, 7 - EtOAc extract of MT_2) (EtOAc – ethyl acetate; MT2 - Mother tincture2)

Plate 5. HPTLC profile of ethyl acetate extracts of plant, Mother tincture2 and standard.

Table 12. HPTLC profile of ethyl acetate extract of commercial formulations

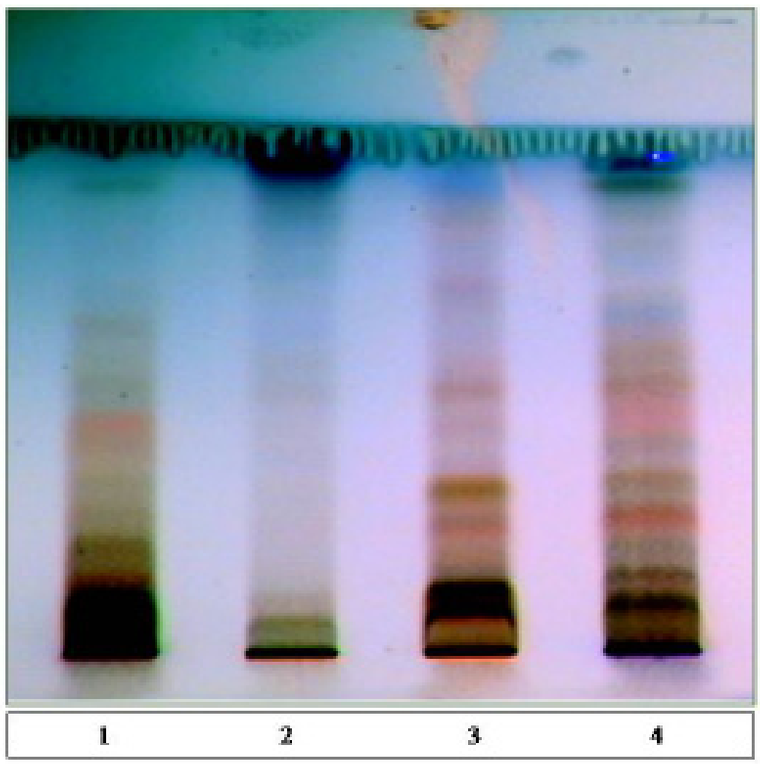
Plate	Track No	Sample	Peaks	Rf value at max	Height of peak at max	AUC of Peak
4	1,2,3	Poly herbal formulation. (Deep act OS)	1	0.12	46.3	1446.1
			2	0.40	65.7	4121.0
			3	0.52	67.8	3404.1
			4	0.55	118.0	3746.7
			5	0.63	97.5	3521.3
3	5,6,7	Mother tincture 1	1	0.51	25.7	1211.3
			2	0.62	60.0	2399.9
			3	0.68	21.2	634.6
5	5,6,7	Mother tincture 2	1	0.52	26.23	1646.7
			2	0.60	32.75	1840.6
			3	0.66	41.02	1585.4
5	4	Caffeic acid	1	0.52	85.0	4176.9

quantitation of β- sitosterol and β- amyrin was done in n hexane extract and caffeic acid in ethyl acetate extracts using standard plots of β-sitosterol, β-amyrin and caffeic acid.

Development of calibration curves

a) β-Sitosterol (0.1 mg/ml): Aliquots of stock solution - 3 µl, 5 µl, 7 µl, 9 µl, 11 µl, 13 µl corresponding to 0.3 µg, 0.5 µg, 0.7 µg, 0.9 µg, 1.1 µg, 1.3 µg of β-sitosterol were applied on precoated silica gel 60 TLC plate. The plate was developed in mobile phase (Toluene: ethyl acetate, 8:2) and scanned at 560 nm.

b) β-Amyrin (0.1 mg/ml): Aliquots of stock solution - 1 µl, 3 µl, 5 µl, 7 µl, 9 µl corresponding to 0.1 µg, 0.3 µg, 0.5 µg, 0.7 µg, 0.9 µg of β-amyrin were applied on



(1 - MeOH extract of plant; 2 - MeOH extract of PHF; 3 - MeOH extract of MT_1; 4 - MeOH extract of MT_2) (MeOH – methanol; PHF - poly herbal formulation; MT1 - Mother tincture 1; MT2 - Mother tincture 2)

Plate 6. HPTLC profile of methanol extract of plant and commercial formulations.

Table 13. HPTLC profile of methanol extract of plant leaves and commercial formulations

Track No	Sample	Peaks	Rf value at max	Height of Peak at max	AUC of Peak
1	Leaves	1	0.01	91.5	3400.9
		2	0.12	38.9	970.7
		3	0.34	14.2	533.3
		4	0.43	17.5	488.0
		5	0.86	25.1	466.9
2	PHF	1	0.02	13.5	199.7
		2	0.45	12.2	504.5
3	MT 1	1	0.02	255.6	4993.7
		2	0.03	211.2	4508.7
		3	0.17	47.1	1463.5
		4	0.24	87.0	2158.1
		5	*0.36*	15.0	408.3
		6	0.44	24.8	787.4
		7	0.66	16.2	629.5
		8	0.87	22.4	477.9
4	MT 2	1	0.01	121.8	3805.8
		2	0.06	88.8	960.5
		3	0.12	22.0	396.2
		4	0.19	55.3	1347.4
		5	0.26	42.4	911.7
		6	0.33	26.5	673.8
		7	0.45	51.3	2407.2
		8	0.51	39.5	838.9
		9	0.60	60.6	2617.5
		10	0.73	14.3	501.8
		11	0.86	79.3	1621.8

PHF- Poly herbal formulation, MT1- Mother tincture 1, MT2- Mother tincture 2.

precoated silica gel 60 TLC plate. The plate was developed, derivatized, scanned as similar as β-sitosterol.

c) Caffeic acid (0.01 mg/ml): Aliquots of stock solution (10 µl × 4 times), (20 µl × 2 times), corresponding to 0.1 µg, 0.2 µg of caffeic acid were applied on precoated silica gel 60 TLC plate. The plate was developed in mobile phase (Toluene: EtOAc: acetic acid, 5:4:1) and scanned by UV 366 nm.

The calibration curves for different standards were studied by linear regression method. Quantitative analysis of markers in different extracts was carried out. The amount of β- sitosterol and β-amyrin was determined using calibration of curve of standards and area under the curve (AUC) of the n- hexane extract of plant and in PHF and the amount of Caffeic acid in ethyl acetate extracts of plant and in commercial formulations was determined using AUC of plant, MT_1, PHF and MT_2 (Tables 14–16).

The study has revealed the quantities of marker compounds in different extracts of plant and commercial formulations (Table 17). The commercial formulations studied in this study contained the marker compounds in quantities greater than that of the plant extracts.

CONCLUSIONS

HPTLC method for qualitative evaluation and quantitative estimation of marker compounds in extracts was found to be simple, precise, specific, and sensitive and can be used for routine quality control of plant material and commercial formulation of the plant.

ACKNOWLEDGEMENTS

Authors thankfully acknowledge the facilities provided by the Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala.

Table 14. Analysis of n-hexane extracts

Sample	Qualitative Analysis (presence/absence)		Quantitative Analysis Mean(%w/w of marker) ± S.D.	
	β- sitosterol	β- amyrin	β- sitosterol	β- amyrin
Leaves	+	+	0.021 ± 0.0017	0.016 ± 0.0007
PHF	+	+	0.133 ± 0.0043	0.046 ± 0.0015
MT1	–	–	–	–
MT2	–	–	–	–

PHF- Poly herbal formulation, MT1- Mother Tincture 1, MT2- Mother tincture 2.

Table 15. Analysis of ethyl acetate extracts

Sample	Qualitative Analysis (presence of caffeic acid)	Quantitative Analysis Mean (% w/w of caffeic acid) ± S.D.
Leaves	+	0.013 ± 0.0015
PHF	+	0.020 ± 0.0007
MT1	+	0.013 ± 0.001
MT2	+	0.010 ± 0.0007

PHF- Poly herbal formulation, MT1- Mother tincture 1, MT2- Mother tincture 2.

Table 16. Qualitative analysis of methanol extracts

Sample	Number of Peaks	Peak at Rf 0.3	Peak at Rf 0.43
Leaves	5	Present	Present
PHF	2	Absent	Present
MT1	8	Present	Present
MT2	11	Present	Present

PHF- Poly herbal formulation, MT1- Mother tincture 1, MT2- Mother tincture 2.

Table 17. Quantitative analysis of various extracts

Sample	Percentage of marker compounds in prepared extracts		
	n-hexane extract		Ethyl acetate extract
	β -Sitosterol	β -Amyrin	Caffeic acid
N. arbortristis	0.021 ± 0.0017	0.016 ± 0.0007	0.013 ± 0.0015
PHF	0.133 ± 0.0043	0.046 ± 0.0015	0.020 ± 0.0007
Mother tincture 1	–	–	0.013 ± 0.001
Mother tincture 2	–	–	0.010 ± 0.0007

PHF poly herbal formulation.

REFERENCES

1. Kirtikar KR , Basu BD. Indian Medicinal Plants, 2nd edition, Allahbad, India; 1935; 2:1526–1528.
2. Zaheer SH, Prasad B, Chopra RN, Santapau H, Krishnan MS. The Wealth of India. A dictionary of Indian raw material and industrial products. CSIR, New Delhi; 1966; 7:69–70.
3. Jayaweera DMA. Medicinal Plants. Colombo, National Science Council of Sri Lanka. 1981; 151.
4. Yusuf M, Choudhury JU, Wahab MA, Begum J, Medicinal Plants of Bangladesh. BCBIR Laboratory, Chittagong, Bangladesh.1994.
5. Dymock W, Warden CJH, Hooper D.A history of the Principal drugs of vegetable origin. Pharmacographia Indica. 1891; 2:376–378.
6. Partomihardjo T, Nyctanthes arbortristis L, In lemmens RHMT, Wulijarni soetjipto N, (eds). Plant resources of South East Asia. No, 3: Dye and tannin producing plants. Prosea foundation, Bogor, Indonesia. 1992; 97–99.
7. Jain SK, Tarafder CR. Medicinal plant-lore of the santals (A revival of P.O.Bodding's work). Econ Bot.1970; 24:241.
8. Jain SK. Medicinal plant-lore of the tribals of Bastar. Econ Bot, 1965; 19:236.
9. Drury CH. The useful plants of India.2nd edn. Allen, W.H.and co, London. 1873; 314.
10. Charak, Samhita, Shri Gulabkunverba Ayurvedic Society, Ayurvedic muranlaya, Jamnagar. 1949; 5:23–24.
11. Chopra RN, Chopra IC, Varma BS, Supplement to glossary of Indian medicinal plants. CSIR, New Delhi, 1959; 63–64.
12. Chopra RN, Chopra IC, Handa KL, Kapur LD, Chopra's Indigenous drugs of India.2nd edn. Dhar UN and sons, Calcutta, 1958; 408.
13. Nadkarni KM, Indian Materia Medica, Popular prakashan, Bombay, India, 1976; 1:857.
14. Nagendrappa PB, Naik MP, Payyappallimana U, Ethnobotanical survey of malaria prophylactic remedies in Odisha, India. J Ethnopharmacol. 2013; 1463:768–72.
15. Gupta AK, Tandon N, Sharma M, Quality standards of Indian medicinal plants, volume 3, Indian Council of Medical research (ICMR), New Delhi, India, 2005; 3:281–289.
16. Sah AK, Verma VK, Phytochemicals and Pharmacological Potential of Nyctanthes arbortristis: A Comprehensive Review. International Journal of Research in Pharmaceutical and Biomedical Sciences 2012; 31:420–426.
17. Priya K, Ganjewala D, Antibacterial activities and phytochemical analysis of different plant parts of *Nyctanthes arbor-tristis* (Linn.). Research Journal of Phytochemistry, 2007; 1:1–7.
18. Rathee SJ, Hassarajani SA, Chattopadhyay S, Antioxidant activity of *Nyctanthes arbor-tristis* leaf extract. Food chemistry, 2007; 1034:1350–1357.
19. Srivastava AK, Khare RL, Udadhyay RK, Jha AK, Dangi JS, Talwar N, Some pharmacological studies on a flavone glycoiside of Nyctanthes arbortristis (harsinghar). Anc Sci Life, 1991; 104:245–7.
20. Anjaneyulu ASR, Murty YLN, Row LR, The triterpenoid constituents of the leaves of *Nyctanthes arbortristis* Linn. J. Indian Chem Soc. 1981; 58:817–818.
21. Singh KL, Roy R, Srivastava V, Tandon JS, Mishra A, Arborside D, A minor Iridoid glucoside from *Nyctanthes arbortristis.* J. of Natural products, 1995; 58:1562–1564.
22. Rimpler H, Jinghanns JU, Nyctanthosid, ein neues iridoid aus *Nyctanthes arbortristis* Linn. Tetrahedron Letters, 1975; 1629:2423–2424.
23. Rathore A, Juneja RK, Tandon JS, An iridoid glucoside from *Nyctanthes arbortristis.* Phytochemistry, 1989; 287:1913–1917.
24. Srivastava V, Rathore A, Ali SM, Tandon JS, New benzoic esters of loganin and 6β-hydroxy loganin from *Nyctanthes arbortristis.* Journal of Natural Products, 1990; 532:303–308.
25. Tandon JS, Srivastava V, Guru PY, Iridoids: A new class of Leshmancidal agents from *Nyctanthes arbortristis.* J. of Natrual Products, 1991; 544:1102–1104.
26. Stuppner H, Muller EP, Mathuram V, Kundu AB, Iridoid glucosides from *Nyctanthes arbortristis.* Phytochemistry, 1993; 32:375–378.
27. Mathuram V, Kundu AB, Banerjee S, Patra A, Occurrence of two new esters of 6 beta- hydroxyloganin in *Nyctanthes arbortristis*. J.Indian Chem Soc. 1991; 68:581–584.
28. Sen AB, Singh SP, Chemical examination of *Nyctanthes arbortristis* Linn. J.Indian Chem Soc, 1964; 41:192–194.
29. Biswas I, Ukil S, Mukherjee A, Determination of n-alkane constituents and their phenological variation in the epicuticular wax of mature leaves of Nyctanthes arbor-tristis L. Nat Prod Res. 2013 Oct 21. [Epub ahead of print].
30. Chandra G, Chemical composition of the flower oil of *Nyctanthes arbortristis* Linn. Indian Perfum. 1970; 141: 19.
31. Tuntiwachwuttikul P, Rayani, K, Taylor WC, Chemical constituents from the flowers of *Nyctanthes arbortristis.* Science Asia, 2003; 29:21–30.
32. Dhingra VK, Seshadri TR, Mukerjee SK, Carotenoid glycosides of *Nyctanthes arbortristis* Linn. J. Indian Chem Soc, 1976; 14: 231.
33. Khatune NA, Mossadik MA, Rahman MM, Khonkar P, Haque ME, Gray AI, A Benzofuranone from the flowers of *Nyctanthes arbortristis* and its Antibacterial and cytotoxic activities. Pharmaceutical Science Journal, 2005; 41:33–37.
34. Gadgoli C, Shelke S, Crocetin from the tubular calyx of *Nyctanthes arbor-tristis*. Nat Prod Res. 2010; 2417:1610–5.
35. Purushothaman KK, Mathuram V, Sarada A, 1985. Arbortristoside A and B two iridoid glucosides from *Nyctanthes arbortristis.* Phytochemistry, 1985; 244:773–776.
36. Khatune NA, Mosaddik MA, Haque ME, Antibacterial activity and cytotoxicity of *Nyctanthes arbortristis* flowers. Fitoterapia, 2001; 72:412–414.
37. Saxena RS, Gupta B, Saxena KK, Singh RS, Prasad DMJ, Study of anti-inflammatory activity in the leaves of *Nyctanthes arbor-tristis* Linn.--an Indian medicinal plant. J. of Ethnopharmacology, 1984;113:319–330.
38. Saxena RS, Gupta B, Saxena KK, Srivastava VK, Prasad DN, Analgesic, antipyretic and ulcerogenic activity of *Nyctanthes arbortristis* leaf extract. J. of Ethnopharmacology, 1987;192:193–200.
39. Paul BN, Saxena AK, Depletion of tumor necrosis factor-alpha in mice by *Nyctanthes arbor-tristis*. J. of Ethnopharmacology, 1997; 562:153–158.
40. Paul BN, Prakash A, Kumar S, Yadav AK, Mani U, Saxena AK, Sahu AP, Lal K, Dutta KK, Silica induced early fibrogenic reaction in lung of mice ameliorated by *Nyctanthes arbortristis* extract. J. of Biomedical and Environmental Sciences, 2002; 15:215–222.
41. Saxena RS, Gupta B, Lata S, Tranquilizing, antihistaminic and purgative activity of *Nyctanthes arbor-tristis* leaf extract. J. of Ethnopharmacol. 2002; 81:321–325.
42. Talakal TS, Dwivedi SK, Sharma SRJ, In vitro antitrypanosomal, potential of *Nyctanthes arbor-tsristis* leaves. Pharm. Biol. 2000; 385:326–329.
43. Sopi RB, Hayat Khan MF, Bronchodilatory effect of ethanolic extract of the leaves of *Nyctanthes arbortristis*. Pharmacognosy Res. 2013; 53:169–72. doi: 10.4103/0974-8490.112422.
44. Verma N, Kaur J, Bhatia A, Stimulation of acetylcholinesterase activity with N*yctanthes arbor-tristis* leaves extract in the malathion-treated immunosuppressed mice. Intern. J. Environ. Studies, 2001; 58:645–654.
45. Deshmukh VS, Juvekar AR, Antistress anixiolytic and nootropic activity of *Nyctanthes arbortristis* leaves. Planta Med. 2006; 72–S_019
46. Michael JS, Kalirajan A, Padmalatha C, Singh AJ, In vitro antioxidant evaluation and total phenolics of methanolic leaf extracts of *Nyctanthes arbor-tristis* L. Chin J Nat Med. 2013 Sep;115:484–7. doi: 10.1016/S1875-5364(13)60088-6.

47. Dasgupta N, De B, Antioxidant activity of some leafy vegetables of India: A comparative study. Food chemistry, 2007; 101: 471–474.
48. Meghashri S, Gopal S, Biochemical characterization of radical scavenging polyphenols from *Nyctanthes arbortristis*. J Pharm Bioallied Sci. 2012; 44:341–4. doi: 10.4103/0975-7406.103277.
49. Hukkeri VI, Kusum SA, Sureban RR, Gopalakrishna B, Byahatti VV, Rajendra SV, Hepatoprotective activity of the leaves of *Nyctanthes arbor-tristis* linn.Indian Journal of Pharmaceutical Sciences, 2006; 68:542–543.
50. Nirmal SA, Pal SC, Mandal SC, Patil AN, Analgesic and anti-inflammatory activity of β-sitosterol isolated from *Nyctanthes arbortristis* leaves. Inflammopharmacology, 2012; 204:219–24.
51. Kumari P, Sahal D, Jain SK, Chauhan VS, Bioactivity guided fractionation of leaves extract of *Nyctanthes arbor-tristis* (Harshringar) against *P falciparum*. PLoS One. 2012; 712:e51714. doi: 10.1371/journal.pone.0051714. Epub 2012 Dec 26.
52. Agrawal J, Shanker K, Chanda D, Pal A, Nyctanthes arbor-tristis positively affects immunopathology of malaria-infected mice prolonging its survival. Parasitol Res. 2013; 1127:2601–9. doi: 10.1007/s00436-013-3427-y. Epub 2013 Apr 30.
53. Gupta RS, Kachhawa JBS, Sharma R, Antispermatogenic effects of *Nyctanthes arbortristis* in male albino rats. Pharmacology online, 2006; 2:261–273.
54. Omkar A, Jeeja T, Chhaya G, Evaluation of anti-inflammatory activity of *Nyctanthes arbortristis* and *Onosma echioides.* Pharmacognosy magazine, 2006; 28:258–260.
55. Khatune NA, Islam ME, Rahman MAA, Mosaddik MA, Haque ME, In Vivo cytotoxic evaluation of a new benzofuran derivative isolated from *Nyctanthes arbortristis* Linn on Ehrlich Ascite carcinoma cells (EAC) in mice. J.Med.Sci, 2003; 32:169–173.
56. Khatune NA, Mossadik MA, Haque ME, Laboratory evaluations of *Nyctanthes arbortristis Linn* flower extract and it's isolated compound against common filarial vector, *Culex uinquefasciatus* Larvae. Pakistan Journal of Biological Sciences, 2001; 45: 585–587.
57. Ratnasooriya WD, Jayakody JRAC, Hettiarachchi ADI, Dharmasiri MG, Sedative effects of hot flower infusion of *Nyctanthes arbortristis* on rats. Pharmaceutical Biology, 2005; 432:140–146.
58. Shukla AK, Patra S, Dubey VK, Deciphering molecular mechanism underlying antileishmanial activity of *Nyctanthes arbortristis*, an Indian medicinal plant. J Ethnopharmacol. 2011; 1343:996–8.
59. Shukla AK, Patra S, Dubey VK, Iridoid glucosides from *Nyctanthes arbortristis* result in increased reactive oxygen species and cellular redox homeostasis imbalance in Leishmania parasite. Eur J Med Chem. 2012 May 2.
60. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS, Immunostimulant activity of *Nyctanthes arbortristis* Linn. J. of Ethnopharmacol. 1994; 42:31–37.
61. Gupta D, Bajpai SK, Chandra K, Singh KL, Tandon JS, Antiviral profile of *Nyctanthes arbortristis* L. against encephalitis causing viruses.Indian J. of Experimental Biology, 2005; 4312:1156–1160.
62. Das S, Sasmal D, Basu SP, Anti-inflammatory and antinociceptive activity of arbortristoside-A. J Ethnopharmacol. 2008; 1161: 198–203.
63. Das S, Sasmal D, Basu SP,. Evaluation of CNS Depressant Activity of Different Plant parts of *Nyctanthes arbortristis* Linn. Indian J Pharm Sci. 2008; 706:803–6.
64. Sasmal D, Das S, Basu SP, Diuretic activity of *Nyctanthes arbortristis* Linn. Anc Sci Life. 2007; 272:19–23.
65. Singh SP, Bhttacharji S, Sen AB, Flavanoids of *Nyctanthes arbortristis* Linn. Bull Natl Inst Sci India, 1965; 31:41.