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Original article

Evaluation of antioxidant, anti-inflammatory and adipocyte differentiation inhibitory potential of *Ziziphus mauritiana* bark extract

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ABSTRACT

Introduction: *Ziziphus mauritiana* is a shrubs belonging to family Rhamnaceae and distributed in warm temperature zone from western Africa to India. Earlier, anti-cancer, anti-inflammatory and anti-diabetic activities were attributed to *Z. mauritiana*.
Materials and methods: The antioxidant activity of chloroform, ethanol and aqueous extracts of *Z. mauritiana* bark was assessed by ABTS + decolourization assay. The anti-inflammatory activity was studied in Wistar rats by Carrageenan induced paw edema assay. The adipocyte differentiation (in 3T3-L1 adipocyte cell) and glucose uptake (in CHO-HIRC-mycGLUT4e GFP cells) assay of *Z. mauritiana* bark aqueous extract was performed.
Results: The chloroform, ethanol and aqueous extracts showed good antioxidant activity. The anti-inflammatory activity (27.83% reduction in inflammation) was observed with chloroform extract at the concentration of 200 mg/kg body weight when administered orally in rats. The aqueous extract showed reduction in number and size of the oil droplets in 3T3-L1 adipocyte cell cytoplasm which was found to be dose dependent. The aqueous extract stimulated the glucose uptake in the CHO-HIRC-mycGLUT4e GFP cells which was comparable to insulin.
Conclusion: The chloroform, ethanol and aqueous extracts showed good antioxidant activity. The anti-inflammatory potential was shown by chloroform extract only. The adipocyte differentiation inhibition and glucose uptake in CHO-HIRC-mycGLUT4e GFP cells was induced by aqueous extract.

1. Introduction

On a global scale, obesity has reached epidemic proportions and is a major contribution to the global burden of chronic disease such as type 2 diabetes, cardiovascular complications and disability.[1]

Two different types of the drugs are currently available in the market for the treatment of obesity. One of these is Orlistat (xenical) and other is sibutramine (reductil). Orlistat reduces intestinal fat absorption through inhibition of pancreatic lipase, whereas Sibutramine is an appetite suppressant.[2,3] Both these drugs have side effects, including increased blood pressure, dry mouth, constipation, headache and insomnia. A number of anti-obesity drugs are currently undergoing clinical development, including centrally acting drugs, drugs targeting peripheral episodic satiety signals, drugs blocking fat absorption and human growth hormonal fragments.[1]

A variety of natural products also have been proposed as pharmacological treatments for obesity preferably as many synthetic drugs are potentially toxic and are not free of side effects. Several medicinal plants have been reported to induce body weight reduction and prevent diet induced obesity.[4] Amongst these plants, *Z. mauritiana* and *Ziziphus jujuba* are shrubs belong to family Rhamnaceae distributed in warm temperature zone from western Africa to India. Earlier, anti-cancer, anti-inflammatory and anti-diabetic activities were attributed to *Z. mauritiana*.[5–7] Recently anti-obesity activity of *Z. jujuba* leaf extract has been demonstrated in rats fed on high fat diet.[8] In previous studies *Z. mauritiana* bark powder did show weight reduction and loss of peritoneal fat pads in high fat diet induced obese rats.[9] In the present studies we have standardized bioassays to determine antioxidant, anti-inflammatory and lipase inhibition activity of different extracts of bark of *Ziziphus mauritiana*. The aqueous extract was evaluated for its adipocyte differential activity and insulin mimetic activity in vitro assays.

2. Material and methods

2.1. Chemicals

The ABTS, IBMX, Insulin, Dexamethasone, Cerulenin, Glucose and Carrageenan were obtained from Sigma–Aldrich Chemical Co.,

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St. Louis, USA. All the other chemicals used were of Analytical grade and purchased from local supplier. Tritiated 2-DG (15 Ci mmol_1) was obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). DMEM and FBS, were purchased from Life Technologies Inc. (Rockville, MD, U.S.A.). Cultures of 3T3-L1 preadipocytes (ATCC no. CL-173) were obtained from National Centre for Cell Science, Pune, India.

2.2. Animals

Male Wistar rats (100–150 g) were used to determine anti-inflammatory activity of chloroform, ethanol and aqueous extract of bark of *Ziziphus mauritiana*. The animals were maintained under standard laboratory conditions at an ambient temperature of 22 ± 2 °C having $50 \pm 5\%$ relative humidity with 12 h light and dark cycle. Animals were fed a standard laboratory diet with water *ad libitum*.

The Institutional Animal Ethical Committee approved the experimental protocols as per CPCSEA guidelines through the research project no. 81.

2.3. Plant materials

Z. *mauritiana* (ZM) bark was collected in the month of November 2010 from a full grown tree in the Solapur District of Maharashtra state, India and was authenticated by Botanical Survey of India, Pune under the certificate no. BSI/WRC/Tech./2011. The bark was cleaned, dried and ground to a coarse power with the help of pulverizer.

2.4. Preparation of extracts

The coarse ZM bark powder (ZMBP) was kept in Soxhlet apparatus. Chloroform, ethanol and aqueous extracts were obtained by serial extraction with respective solvents. The extracts obtained were concentrated in rotary evaporator under vacuum and their percent yields were determined.

2.5. Phytochemical study

Qualitative analysis of ZMBP extracts for the presence of flavonoids, saponins, tannin, steroids was carried out as per the method described by Khandelwal, 2008.[10]

2.6. Determination of antioxidant potential by in vitro ABTS + decolonization assay

Antioxidant effect of extracts was studied using ABTS (2,2′-azino-bis-3ethylbenzthiaoline-6-sulphonic acid) radical cation decolorization assay according to the method of Re et al, 1999.[11] ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulphate. The mixture was allowed to stand in dark at room temperature for 12–16 h before use. The absorbance was read at 735 nm in a spectrophotometer. The percent inhibitory activity of the plant extract was determined by the following formula and the results were compared with ascorbic acid as standard.

$$\%\ \text{inhibition} = \frac{\text{Abs control} - \text{Abs test}}{\text{Abs Control}} \times 100$$

The concentration equivalent to ascorbic acid was calculated by plotting the values of the test extracts on the standard curve of ascorbic acid.

2.7. Determination of anti-inflammatory potential of ZMBP extracts

The assay based on ability of anti-inflammatory agent to inhibit the edema produced in the hind paw of the rat after injection of carrageenan (phlogistic agent).[12]

Male Wistar rats weighing around 100 g–150 g were used for this assay. Five groups of six animals each were starved overnight. Group 1 Control, group 2 Standard drug Indomethacin (10 mg/kg body weight), group 3, group 4 and group 5 animals administered chloroform, ethanol and aqueous extracts orally at a dose of 200 mg/kg body weight respectively. One hour after dosing, the rats were challenged by a subcutaneous injection of 0.2 ml of 1% solution of carrageenan into the sub planter region to induce edema. The paw edema was measured with a vernier caliper before carrageenan injection and then at 0, 1, 2, 4 and 6 h after carrageenan injection. The difference between the initial and subsequent values gave the actual edema which was compared with the control animals. The present inhibition of inflammation was calculated using the following formula.

$$\%\ \text{inhibition} = \frac{C - T}{C} \times 100$$

where C represents mean edema in Control and T represents mean edema in group treated with standard drug or test drug.

2.8. Adipocyte differentiation assay

In brief, 3T3-L1 preadipocyte were seeded as 5×10^3 cells/well in 24 well plate. After reaching the confluence (48 h) the preadipocytes were induced by IBMX + insulin (10 mg/ml) medium to differentiate to adipocytes for another 48 h. The aqueous extract of ZMBP (ZBEaq) and Cerulinin (a known anti-differentiating agent) were added at different concentration and incubated at 37 °C under humidified 5% CO_2 atmosphere. The medium was then replaced every 2 days with DMEM containing 10% FBS and 5 μg/ml insulin. On day 6, the medium was completely removed for triglyceride estimations. Formaldehyde was added slowly to each well and kept for 30 min at room temperature. Formaldehyde was then aspirated and Oil Red O solution (0.5 g/100 μl Isopropanol) was added in each well and incubated for 1 h at room temperature. The stain was removed completely and washed with distilled water twice. After drying the plate, photographs were taken. The Oil Red O stained oil droplets were extracted in Isopropyl alcohol and absorbance was determined spectrophotometrically at 520 nm.[13]

2.9. GLUT4 translocation assay in CHO-HIRC-mycGLUT4e GFP cells

CHO-HIRC-mycGLUT4e GFP cells (1×10^4 cells/well) were plated in 35 mm plates and serum starved by incubating them in F-12 medium containing 1 mg/ml BSA for 3 h, before addition of insulin or ZBEaq for 10 and 30 min, respectively. GLUT4 translocation was visualized by tracking the movement of GLUT4 associated GFP fluorescence from perinuclear space to the plasma membrane by employing cooled charge coupled device (CCD) camera attached to fluorescent microscope.[14]

2.10. Effect of ZBEaq on glucose transport in CHO-HIRC-mycGLUT4e GFP cells

3H-2-deoxy-D-glucose (2-DG) uptake was measured as described by Vijaykumar 2005.[15] In brief, CHO-HIRC-mycGLUT4e GFP cells (2×10^5 cells/well) grown in 12 well plate were treated with insulin or vehicle control or ZBEaq for 10 and 30 min, respectively. The glucose uptake was initiated by adding 0.1 mM 2-deoxy glucose

Table 1
Phytochemical analysis.

Name of the extract	Phytochemical constituents			
	Steroids	Tannins	Saponins	Flavonoids
Chloroform extract	+	−	−	−
Ethanol extract	−	+	+	+
Aqueous extract	−	+	+	+

containing 0.5 µl/ml of labeled 2-DG for 10 min at 37 °C. The reaction was terminated by keeping the cells on ice and washing 3 times with ice cold PBS containing 20 mM 2D glucose. The cells were solubilized with 0.1% SDS sodium dodecyl sulfate. The lysate was transferred on blotting paper and allowed to dry at 37 °C overnight. Scintillation fluid (1CN) 1.0 ml was added and radioactivity incorporated into cells was quantified in a Liquid Scintillation counter (Packard, Albertville, MN USA).[14]

2.11. Statistical analysis

The data expressed as mean ± SD. Statistical comparisons made using student *t*-test and *p*-value ≤0.05 were considered significant.

3. Results

3.1. Phytochemical analysis

The Phytochemical analysis of various extracts of *Z. mauritiana* bark is summarized in Table 1. The ethanol and aqueous extracts showed apparently similar compositions of tannins, saponins and flavonoids.

3.2. Antioxidant activity of ZMBP extracts

The rate of decolorisation of the bluish green $ABTS^+$ radicals revealed the antioxidant content in the plant extract which was calculated and compared with standard ascorbic acid. The chloroform, ethanol and aqueous extract of bark of *Z. mauritiana* all showed appreciable inhibition of $ABTS^+$ radical cation as shown in Fig. 1.

3.3. Anti-inflammatory activity of ZMBP extracts

At 6 h, chloroform extract showed better anti-inflammatory activity, i.e. 27.83% inhibition as compared to the control group. Inhibition values obtained were comparable with Indomethacin a known anti-inflammatory agent i.e. 34.32% inhibition. Anti-inflammatory activity of ethanol and aqueous extracts of ZMBP did not show appreciable reduction in paw edema of treated rats (Table 2).

3.4. Adipocyte differentiation in the presence of ZBEaq

Preadipocyte (3T3-L1) cells differentiate into adipocytes upon addition of IBMX + insulin induction medium. When ZBEaq was added to the adipocyte culture in the presence of induction medium, preadipocytes remained largely undifferentiated as shown by Oil Red O staining of the treated cells which was comparable to the Cerulinin (Fig. 2a and b). Number and size of the oil droplets in cell cytoplasm were reduced in presence of ZBEaq, which was found to be dose dependent.

3.5. GLUT4 translocation and ZBE induced glucose uptake

In subsequent studies, ZBEaq found to induce GLUT4 translocation as comparable to insulin in CHO-HIRC-mycGLUT4e GFP cells. ZBEaq stimulation of cells expressing GLUT4 transfer from internal compartment to peripheral membrane with resultant increase in the rate of glucose transport was observed as evident in Fig. 3.

4. Discussion

A variety of natural products including crude extracts and isolated compounds from plants, can induce body weight reduction and prevent diet induced obesity.[4] These natural products exhibited many potential anti-obesity mechanisms such as a) lipase inhibitory effect[16] b) suppressive effect on food uptake[17] c) stimulatory effect on energy expenditure[18] d) inhibitory effect on adipocyte differentiation[19] e) regulatory effect on lipid metabolism.[20]

Earlier we have reported anti-obesity activity of *Z. mauritiana* bark in high fat diet induced obese rats by reducing body weight gain, increasing fecal fat mass and decreasing insulin resistance.[9] In the present studies we have carried out extraction of *Z. mauritiana* bark powder using different solvents i.e. chloroform, ethanol and water. Phytochemical analysis showed that chloroform extract contains mainly steroids whereas ethanol and aqueous extract contains tannins as catechin, saponins and flavonoid as quercetins in different amounts. All three extracts showed appreciable antioxidant activities in ABTS + radical cation assay. Chloroform extract of *Z. mauritiana* bark also showed appreciable anti-inflammatory activity. The ethanol and aqueous extracts did not show an anti-inflammatory activity.

Dietary fat is not directly absorbed by the intestine unless fat has been subjected to the action of pancreatic lipase.[1] Therefore pancreatic lipase is one of the most widely studied mechanisms for determining anti-obesity potential of any natural product. In our

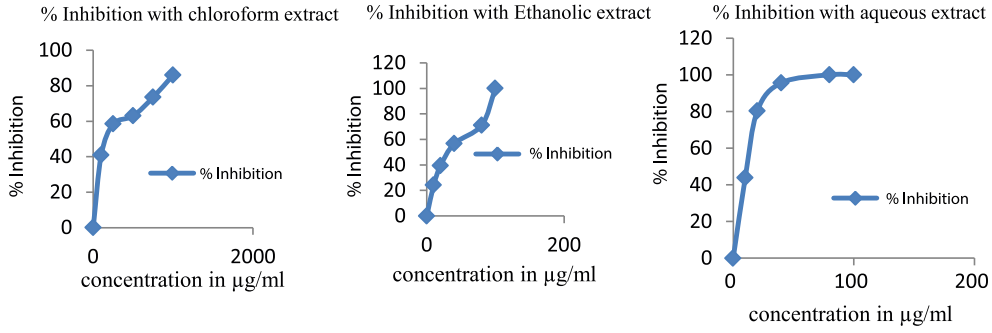


Fig. 1. Antioxidant activity of ZMBP extracts.

Table 2
Anti-inflammatory activity of ZMBP extracts. Data expressed as % inhibition.

Group		Paw (edema) thickness in mm						% inhibition in paw edema at 6 h compared to control
		Before carrageenan injection	After carrageenan injection					
			Immediate	1 h	2 h	4 h	6 h	
1 Control	Mean	4.08	6.06	6.79	7.68	7.73	9.03	
	±SD	0.41	0.33	0.55	0.79	0.64	0.74	
2 Chloroform	Mean	3.74	5.56	6.39	6.82	6.67	7.31*	27.83
	±SD	0.34	0.89	0.49	0.37	0.62	0.32	
3 Ethanol	Mean	3.66	5.81	5.97	7.57	7.80	8.31	6.06
	±SD	0.17	0.75	0.77	0.79	0.62	0.96	
4 Aqueous	Mean	4.37	6.22	6.44	7.47	7.97	8.49	16.79
	±SD	0.95	0.48	0.66	0.54	1.13	0.99	
5 Standard	Mean	3.55	4.88	6.06	5.90	5.82	6.80**	34.32
	±SD	0.33	0.80	0.74	0.84	1.13	0.78	

Note: All the powdered extracts were suspended in corn oil as a vehicle.
*Statistically significant at $p < 0.05$.
**Statistically significant at $p < 0.001$.

previous studies *Z. mauritiana* fed obese rats showed significant decrease in pancreatic lipase activity at different dosages.[9]

Adipogenesis is another common feature of obesity in several studies, screening for anti-obesity products have focused on the processes of adipocyte proliferation and differentiation. 3T3-L1 preadipocyte cells are currently used as in vitro model for the study of obesity.[21] While differentiating into adipocytes from pre-adipocytes in presence of IBMX and insulin, the cells accumulate lipids intracellularly in the form of lipid droplets. These oil droplets can be measured by staining with Oil Red O solution. In our studies when ZBEaq was added to the preadipocyte in the presence of IBMX and insulin, preadipocytes remained largely undifferentiated as shown by Oil Red O staining of the treated cells. Oil droplet formation and their sizes were reduced significantly and found to be dose dependent.

Insulin mimetic activity of aqueous extract of *Z. mauritiana* was studied using glucose transporter 4 (GLUT4) translocation to cell membrane leading to glucose uptake using CHO-HIRC-muc-GLUT4eGFP cells. GLUT4 translocation was visualized by tracking the movement of GLUT4 associated GFP fluorescence from perinuclear space to the plasma membrane by employing cooled charge coupled device (CCD) camera attached to fluorescent microscope which ultimately lead to increased glucose uptake.[14] These results indicated that ZBEaq inhibited the preadipocytes differentiation induced by IBMX + insulin. Also it was puzzling that ZBEaq showed insulin like glucose transport stimulatory activity in CHO-HIRC-muc-GLUT4eGFP cells. Similar observations were reported by Liu 2005,[21] where tannic acid was found to stimulate glucose transport and inhibit adipocyte differentiation in 3T3-L1 cells.

Several natural products have been reported earlier to possess single or multifunctional anti-obesity mechanisms including inhibition of lipid and carbohydrate absorption and acceleration of lipid metabolism and energy expenditure by dietary supplementation of *Nelumbo nucifera* leaves extract.[22] Whereas aqueous extract of *Hibiscus sabdariffa* containing mainly anthocyanins has exhibited many potential anti-obesity mechanisms, including anti hyperglycemic effects, plasma cholesterol level reduction, gastric and pancreatic lipase inhibition, thermogenesis stimulation, inhibition

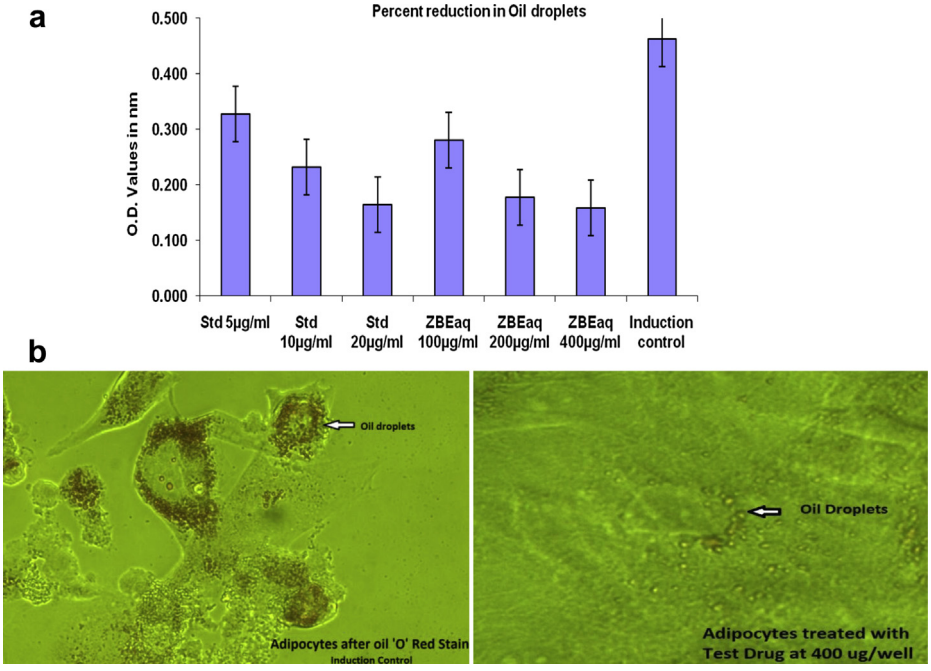
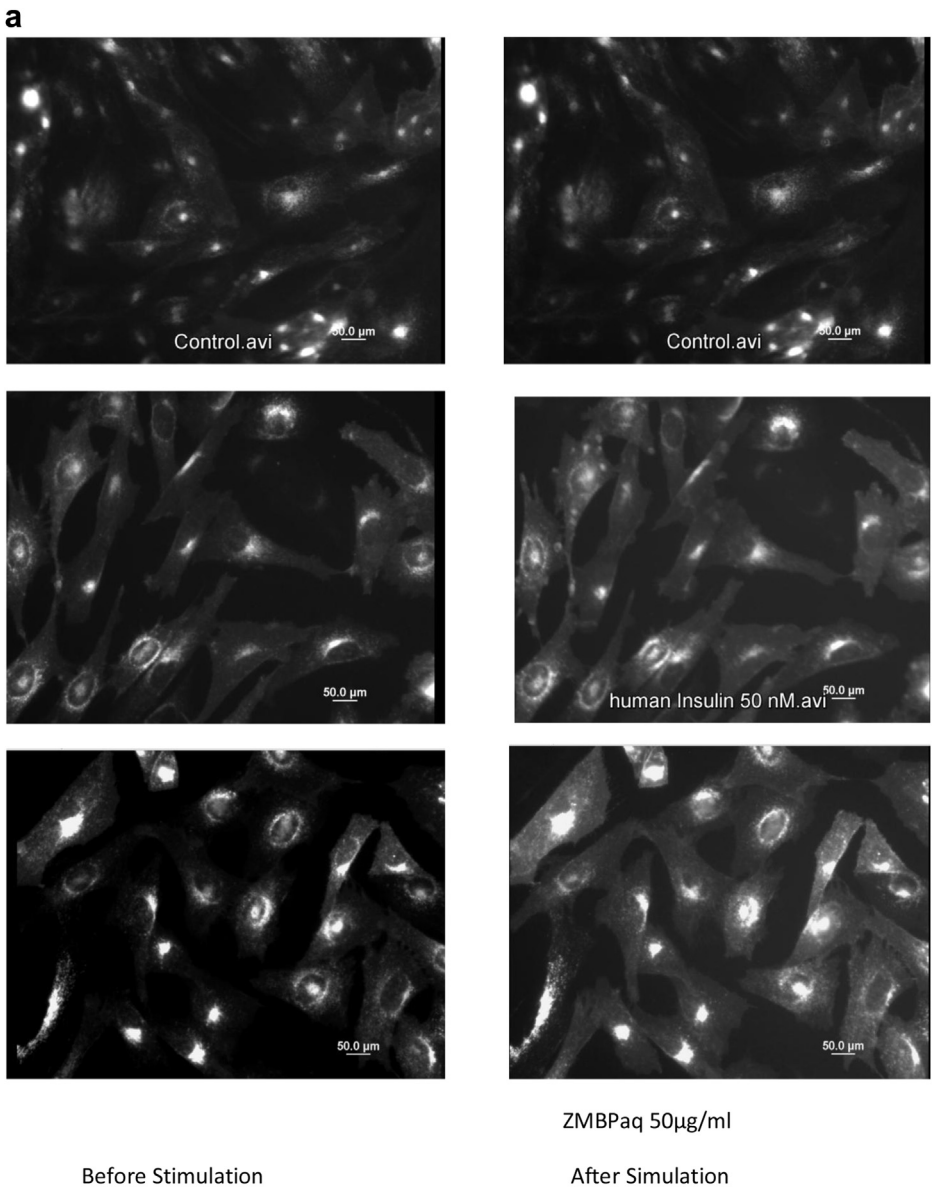


Fig. 2. a: Adipocyte differentiation assays % reduction value. b: Photographs of adipocytes at 40× magnification.



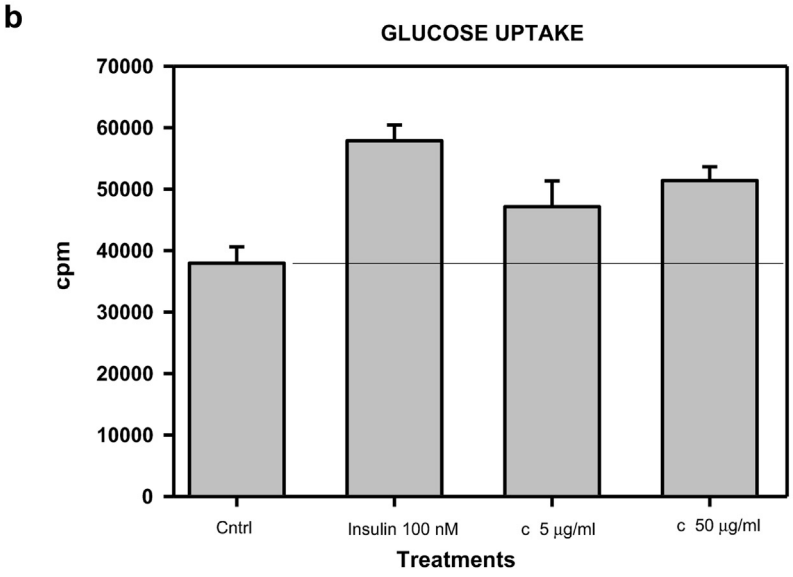


Fig. 3. a: Video image analysis of Glut4 translocation assay. b: Glucose uptake by CHO-HIRC-mycGLUT4e GFP cells.

of lipid droplets accumulation in fat cells and fatty acid synthase inhibition. *Garcinia cambogia* extract (active component, hydroxycitric acid) has also displayed multifunctional activity.[23]

As *Z. mauritiana* aqueous extract is a mixture of tannins saponins and flavonoids, its multifunctional anti-obesity mechanisms such as reduction in weight gain, reduction in insulin resistance, loss of fat through faeces and inhibition of lipid droplet accumulation in fat cells which we have observed earlier together with present studies could be a synergistic effect of mainly tannins and flavonoids.

Conflicts of interest

All authors have none to declare.

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References

1. Yun JW. Possible antiobesity therapeutics from nature – a review. *Phytochemistry*. 2010;71:1625–1641.
2. Drew BS, Dixon AF, Dixon JB. Obesity management: update on Orlistat. *Vasc Health Risk Manag*. 2007;3:817–821.
3. Tzimalos K, Krassas GE, Tzotzas T. The use of Sibutramine in the management of obesity and related disorders: an update. *Vasc Health Risk Manag*. 2009;5:817–821.
4. Nakayama T, Suzuki S, Kudo H, Sassa S, Nomara M, Sakamoto S. Effects of three Chinese herbal medicines on plasma and liver lipids in mice fed a high fat diet. *J Ethnopharmacol*. 2007;109:236–240.
5. Pisha CH, Lee I, Chagwedera YE, et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat Med*. 1995;1:1046–1051.
6. Shiv K, Ganachari MS, Banappa VS. Anti inflammatory activity of *Ziziphus jujube* Lamk. Leaves extracts in rats. *J Nat Remedies*. 2004;4:183–185.
7. Bhatia A, Mishra T. Hypoglycemic activity of *Ziziphus mauritiana* aqueous extract *in alloxan* induced diabetic mice. *Pharm Biol*. 2010;48:604–610.
8. Ganachari MS, Shiv K, Alagawadi KR. Anti-obese activity of *Ziziphus jujube* Lam leaves extract in dietary obese rats. *J Nat Remedies*. 2007;7:102–108.
9. Deshpande M, Shengule S, Apte KG, Wani M, Piprode V, Parab PB. Anti-obesity activity of *Ziziphus mauritiana*: a potent pancreatic lipase inhibitor. *Asian J Pharm Clinl Res*. 2013;6:169–173.
10. Khandelwal KR. Preliminary phytochemical screening. In: *Practical Pharmocognsy*. 19th ed. Nirali Prakashan; 2008:149–156.
11. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol Med*. 1999;26:1231–1237.
12. Winter EA, Risley EA, Nuss GW. Carrageenan induced paw edema in hind paw as an assay for anti inflammatory drugs. *Proc Soc Exp Biol Med*. 1962;111:544–547.
13. Kasturi R, Joshi VV. Hormonal regulation of Stearoyl Coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J Biol Chem*. 1982;257:12224–12230.
14. Vijaykumar MV, Amrendrakumar A, Bhat MK. Demonstration of a visual cell based assay for screening glucose transporter 4 translocation modulators in real time. *J Biosci*. 2010;35:525–531.
15. Vijayakumar MV, Singh S, Chhipa RR, Bhat MK. The hypoglycemic activity of fenugreek seed extract is mediated through the stimulation of an insulin signaling pathway. *Br J Pharmacol*. 2005;146:41–48.
16. Birari RB, Bhutani KK. Pancreatic lipase inhibitors from natural sources: unexplored potential. *Drug Discov Today*. 2007;12:879–889.
17. Van Heerden FR, Marthinus HR, Maharaj VJ, Alegaar R, Senabe JV, Gunning PJ. An appetite suppressant from Hoodia species. *Phytochemistry*. 2007;68:2545–2553.
18. Pang J, Choi Y, Park T. *Ilex papaguareiensis* extract ameliorates obesity induced by high fat diet: potential rate of AMPK in the visceral adipose tissue. *Arch Biochem Biophys*. 2008;476:178–185.
19. Yang JY, Della-Fera MA, Rayalam S, et al. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sci*. 2008;82:1032–1039.
20. Ohkoshi E, Miyazaki H, Shindo K, Watanabe H, Yoshida A, Yajima H. Constituents from the leaves of *Nelumbo nucifera* stimulate lipolysis in the white adipose tissue in mice. *Planta Med*. 2007;73:1255–1259.
21. Liu X, Kim J, Li Y, Li J, Liu F, Chen X. Tannic acid stimulates glucose transport and inhibits adipocyte differentiation in 3T3-L1 cells. *J Nutr*. 2005;135:165–171.
22. Ono Y, Hattori E, Fukaya Y, Imai S, Ohizumi Y. Anti obesity effect of *Nelumba nucifera* leaves extract in mice and rats. *J Ethnopharmacol*. 2006;106:238–244.
23. Alarcon-Aguilar FJ, Zamilpa A, Perez-Garcia MD, et al. Effect of *Hibiscus sabdariffa* on obesity in MSG mice. *J Ethnopharmacol*. 2007;114:66–71.

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Original article

Honokiol reverses depressive-like behavior and decrease in brain BDNF levels induced by chronic corticosterone injections in mice

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ABSTRACT

Background: Honokiol, an active component isolated and purified from Chinese traditional herb *Magnolia officinalis*. It is known to have a wide range of biological activities including antidepressant-like effects which have been observed in stress-induced depression models. This study was designed to investigate the antidepressant potential of honokiol in corticosteroid induced model of depression.
Method: Adult Swiss albino mice were injected with 40 mg/kg of corticosterone (CORT) chronically for 21 days. Behavioral and biochemical parameters were estimated. Moreover, since brain derived neurotrophic factor (BDNF) has been implicated in antidepressant effects of many drugs, we also evaluated the effects of honokiol on BDNF in the hippocampus.
Results: The results showed that the 3-week CORT injections caused the significant elevation in serum CORT levels in mice. Repeated CORT injections also caused depression-like behavior in mice, as indicated by the significant decrease in sucrose consumption ($P < 0.01$) and increase in immobility time in the forced swim test ($P < 0.001$). Moreover, it was found that BDNF levels in the hippocampus were significantly decreased ($P < 0.001$) in CORT-treated mice. Treatment of the mice with honokiol significantly suppressed the depression-like behavior and increased brain BDNF levels ($P < 0.01$) in CORT-treated mice.
Conclusion: These results conclude that honokiol produces an antidepressant-like effect in CORT-induced depression, which is possibly mediated by increasing BDNF expression in the hippocampus.

1. Introduction

Major depression (MD) is a highly prevalent, debilitating and life-threatening psychiatric disorder that affects about 21% of the world population.[1] It is characterized by a persistent low mood, diminished ability to experience pleasure and a variety of other features including anergia, changes in sleep, appetite and suicidal tendency.[2] World Health Organization predicts that depression will be leading cause of disability worldwide by the year 2030.[3] Psychological stress is a common risk factor involved in the development of major depression. Stress associated dysregulation of HPA axis functionality is one of the characteristic features of MD, and is demonstrated by altered feedback inhibition, as seen by increased circulatory cortisol and non-suppression of cortisol following administration of dexamethasone.[4,5] Studies of chronic intense stress and associated glucocorticoid elevation have been performed extensively in adult rodents. Glucocorticoids have been proven to induce depressive-like behavior in rodents, as indicated by the significant changes in behavioral traits, neurochemistry and brain anatomy.[6–8] These findings suggest that a glucocorticoid-induced depression model in rodents is suitable for evaluating the efficacy of potential antidepressants and explore the mechanism of action of antidepressants.[8,9]

At present, there are several types of conventional antidepressants in clinical practice, including tricyclic antidepressants, monoamine oxidase inhibitors, serotonin and noradrenaline reuptake inhibitors. Most of these drugs, however, have undesirable side effects. Thus, there is an unmet need for safe and powerful antidepressants.[10,11] This necessitates the development of safe, better tolerated and effective pharmacotherapeutics, and one such promising class of drugs is plant based natural products.

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Honokiol, a major active component of *Magnolia officinalis* (Magnoliaceae), possesses antioxidant, anti-cancer, anxiolytic, antithrombotic, neuroprotective, antiinflammatory, antidiabetic, antiemetic and antibacterial activities (Fig. 1).[12,13] Previous studies also established that honokiol produces an antidepressant-like effect in chronic stress-induced depression in mice.[14] In this study, the antidepressant-like effect of honokiol treatment was further assessed in a mice model of CORT induced depression. Various other studies have shown that down regulated expression of BDNF is observed in depression, and upon treatment with antidepressants there is an upregulated expression of BDNF in the brain indicating a positive response to therapy.[15–17] we also explored whether the antidepressant-like effect of honokiol was associated with the upregulation of BDNF expression by estimating BDNF levels in hippocampus of mice exposed to CORT.

2. Materials and methods

2.1. Drugs and chemical reagents

Honokiol, fluoxetine hydrochloride, corticosterone and DMSO were purchased from Sigma Aldrich (USA). BDNF ELISA kit purchased from Promega Corporation (Madison, WI) and corticosterone ELISA kit was purchased from Abnova Corporation (Walnut, CA). All the other reagents and chemicals used were of analytical grade.

2.2. Animals

Male Swiss albino mice weighing 20–25 g were obtained from the Pasteur Institute, Shillong, India. The animals were maintained on a 12-hour (h) light/dark cycle under regulated temperature (22 ± 2 °C), humidity (45 ± 10%) and fed with standard diet and water *ad libitum*. They were allowed to acclimatize seven days before use. The animal experiments have been approved by the Institutional Animal Ethics Committee of the Gauhati Medical College, Assam, India. All procedures were conducted in accordance with the CPCSEA guidelines for the care and use of laboratory animals.

2.3. Experimental design

Animals are divided into four groups of eight animals in each group, includes; vehicle control group (saline containing 0.1% dimethyl sulfoxide and 0.1% Tween-80), CORT plus vehicle (positive control) group, CORT plus honokiol (20 mg/kg) group and CORT plus fluoxetine (15 mg/kg) group. CORT (40 mg/kg, dissolved in saline containing 0.1% dimethyl sulfoxide and 0.1% Tween-80) was administrated subcutaneously (s.c.) for 21 days. Honokiol and fluoxetine were administered intragastrically (p.o.) 30 minutes (min) prior to the CORT injection for 21 days. The doses of CORT, honokiol and fluoxetine were selected based on earlier studies.[14,18,19] The experiments were conducted in a noise-free, illumination controlled room. Efforts were made to minimize the number and suffering of the animals.

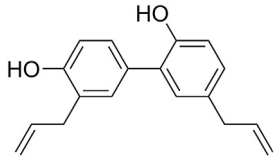

Fig. 1. Chemical structure of honokiol.

2.4. Behavioral tests

After 21-day treatment with respective drugs, mice were randomly assigned to a series of behavioral tests for depression like behavioral characterization. To avoid having compound effect due to sequential behavioral measurements, 24 h later after last CORT treatment sucrose preference test was performed, forced swim test started with a recovery period of 48 h in between.[17]

2.5. Sucrose preference test

The test was performed as described previously,[20] with minor modifications. In this test, mice were given a free choice between two bottles for 24 h, one with 1% sucrose solution and another with regular water. To avoid possible effects of side preference in drinking behavior, the location of the bottles was switched after 12 h. No previous food or water deprivation was applied before the test. The consumption of water and sucrose solution was estimated simultaneously in control and experimental groups by measuring the amount of solution (ml) in the each bottle. The sucrose preference was calculated by the following formula:

Sucrose preference = sucrose consumption (ml)/(water consumption (ml) + sucrose consumption (ml)) × 100.

2.6. Forced swim test

The test was carried out 48 h after the sucrose preference test, according to the method of Porsolt et al.[21] Briefly, mice were forced to swim in a transparent glass vessel (25 cm high, 14 cm in diameter) filled with 10 cm of water at 25 ± 1 °C. The total duration of immobility in seconds (s) was measured during the last 4 min of a single 6-min test session. Immobility period was regarded as the time spent by the mouse floating in the water without struggling and making only those movements necessary to keep its head above the water.

2.7. Biochemical parameters

After 24 h of conducting forced swim test, blood samples were collected by retro orbital method.[22] Serum was separated by centrifugation at 4000× *g* for 10 min and stored at −80 °C until assay. Whole brains were rapidly removed and placed on an ice-chilled glass plate. The hippocampus of animals was dissected on a cold plate and placed in an isolation medium containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4, and homogenized in the same medium. Homogenates were centrifuged at 4000× *g* at 4 °C for 10 min to obtain the supernatant. Aliquots of the supernatant were used for the determination of BDNF levels.

2.8. Estimation of serum CORT levels

Serum corticosterone level was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abnova Corporation, Walnut, CA), according to the manufacturer's protocol. Briefly, 25 μl of standard, sample solutions were added to the already precoated antibody plate provided with the kit and then immediately 25 μl of biotinylated corticosterone was added to each well and incubate for 2 h. After washing 50 μl of Streptavidin–Peroxidase Conjugate was added to each well and incubated for 30 min. After incubation, plate washed properly and 50 μl of chromogen substrate was added and incubated for 12 min. The reaction was stopped with 50 μl of stop solution and absorbance was read at 450 nm immediately. The detection limit of the assay is ~0.3 ng/ml.

2.9. Estimation of BDNF levels

The BDNF content in the hippocampus homogenate measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit from Promega Corporation (Madison, WI) according to the manufacturer's protocol. All samples and standards were added in duplicate into 96-well plate precoated with Anti-BDNF mAb, which were incubated overnight without shaking at 4 °C. After washing, biotinylated anti-Human BDNF pAb were added and the plate was incubated for 2 h at room temperature. After washing, anti-IgY HRP Conjugate solution was added and incubated for 1 h at room temperature with shaking (500 rpm).TMB/E substrate was added and the plate was incubated at room temperature with shaking for 10 min. The reaction was stopped with 100 µl of 1 N hydrochloric acid and then immediately absorbance was read using a microplate reader recorded at 450 nm. The detection limit of the assay is 15.6 pg/ml of BDNF.

2.10. Statistical analysis

All the data were expressed as mean ± S.E.M. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test in order to detect inter-group differences. Difference was considered statistically significant when the $P < 0.05$.

3. Results

3.1. The effect of honokiol on sucrose consumption

The effect of honokiol on the percentage of sucrose consumption in CORT-only treated mice is given in Fig. 2. The CORT injections resulted in a significant reduction in the percentage of sucrose consumption in the animals ($P < 0.01$) compared with the controls. Treatment with honokiol and fluoxetine significantly increased the percentage of sucrose consumption in the CORT-treated mice ($P < 0.05$ and $P < 0.01$, respectively) compared with the CORT-only treated mice.

3.2. The effect of honokiol on immobility time in forced swim test

In this test, animals treated with CORT (40 mg/kg) showed increase in immobility time, which was significant ($P < 0.001$) when compared with vehicle control [Table 1]. Animals treated with fluoxetine (15 mg/kg) showed a significant decrease in the immobility time ($P < 0.001$) as compared to CORT control. Similarly animals treated with honokiol also showed significant decrease in immobility time ($P < 0.01$) and thus effective antidepressant activity.

Table 1
Effects of the honokiol on the duration of immobility in the forced swimming test. All the values are expressed in mean ± S.E.M ($n = 8$); ***$P < 0.001$; **$P < 0.01$ when compared to [a]Vs vehicle control, [b]Vs positive control (CORT).

Groups	Dose (mg/kg)	Immobility period (s) on day 21
Vehicle control	–	83.1 ± 6.52
Positive control (CORT)	40 mg/kg	166.3 ± 11.6*** [a]
CORT + honokiol	20 mg/kg	117.2 ± 8.54** [b]
CORT + fluoxetine	15 mg/kg	97.7 ± 7.42*** [b]

3.3. The effect of honokiol on serum CORT levels

The serum CORT levels in different groups are shown in Fig. 3. The CORT injections resulted in a significant increase in the level of serum CORT in the animals ($P < 0.001$) when compared with the vehicle controls. Treatment with honokiol (20 mg/kg) and fluoxetine (15 mg/kg) significantly decreased serum CORT levels ($P < 0.01$ and $P < 0.01$, respectively) when compared with the CORT-only treated mice.

3.4. The effect of honokiol on BDNF levels in the hippocampus

The effect of honokiol on BDNF levels in the hippocampus of CORT-treated mice is given in Fig. 4. Exposure to CORT significantly decreased BDNF protein levels in the hippocampus ($P < 0.001$) compared with the vehicle controls. Treatment with honokiol (20 mg/kg) and fluoxetine (15 mg/kg) significantly increased BDNF protein levels in the hippocampus ($P < 0.01$ and $P < 0.001$, respectively) when compared with the CORT-only treated mice.

4. Discussion

The corticosteroid induced model of depression is received as a suitable method for predicting the antidepressant action of compounds rodents. In this study, the antidepressant-like effects of

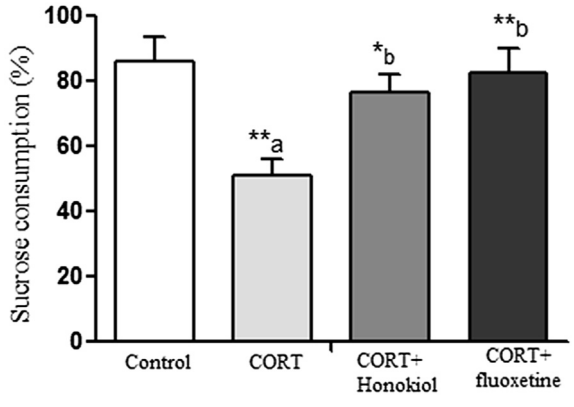

Fig. 2. Effect of honokiol on the percentage of sucrose consumption in CORT-treated mice. The mice were administered with CORT (40 mg/kg, s.c.) once daily for 21 days, and honokiol (20 mg/kg, p.o.) and fluoxetine (15 mg/kg, p.o.) were given to the animals 30 min prior to the CORT injection. Sucrose consumption is the relative volume of sucrose intake when compared with the total liquid intake. All the values are expressed in mean ± S.E.M ($n = 8$); *$P < 0.05$; **$P < 0.01$ when compared to [a]Vs vehicle control, [b]Vs positive control (CORT).

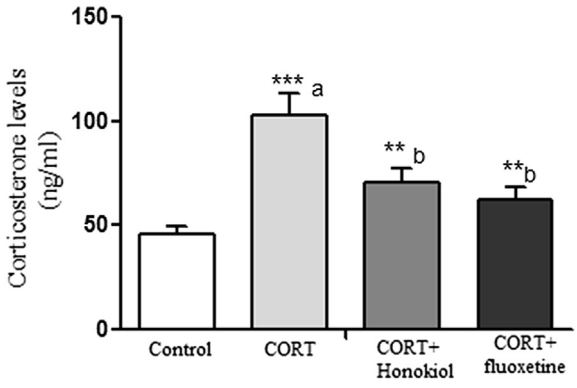

Fig. 3. Effect of honokiol on the serum CORT levels in the CORT-treated mice. The mice were administered with CORT (40 mg/kg, s.c.) once daily for 21 days, and honokiol (20 mg/kg, p.o.) and fluoxetine (15 mg/kg, p.o.) were given to the animals 30 min prior to the CORT injection. All the values are expressed in mean ± S.E.M ($n = 8$); ***$P < 0.001$; **$P < 0.01$ when compared to [a]Vs vehicle control, [b]Vs positive control (CORT).

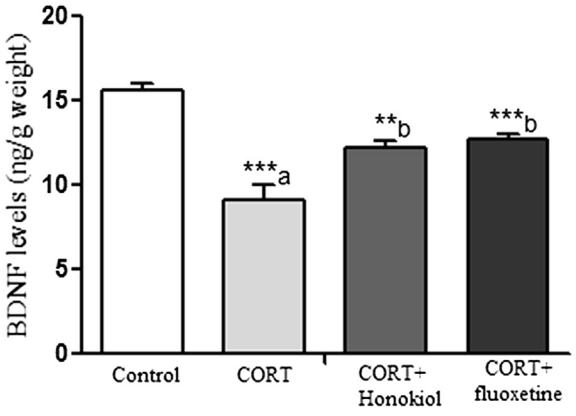

Fig. 4. Effect of honokiol on BDNF protein levels in the hippocampus of CORT-treated mice. The mice were administered with CORT (40 mg/kg, s.c.) once daily for 21 days, and honokiol (20 mg/kg, p.o.) and fluoxetine (15 mg/kg, p.o.) were given to the animals 30 min prior to the CORT injection. All the values are expressed in mean ± S.E.M ($n = 8$); $^{***}P < 0.001$; $^{**}P < 0.01$ when compared to ^{a}Vs vehicle control, ^{b}Vs positive control (CORT).

honokiol were investigated in the corticosteroid induced depression model. Honokiol showed antidepressant-like effect by reversing the depressive-like behavior, decrease in brain BDNF level and increase in corticosteroid levels induced by chronic CORT injections in mice.

The sucrose preference test is an indicator of anhedonia-like behavioral change. Anhedonia, a core symptom of major depression among humans, is modeled by inducing a decrease in responsiveness to rewards, as reflected by the reduced consumption of and/or preference for sweetened solutions. In the present study, our data is in line with other findings showing that repeated CORT injections results in significant decreased in the percentage of sucrose consumption of mice,[18] whereas treatment with honokiol (20 mg/kg) significantly suppressed the percentage of CORT injection-induced decrease in sucrose consumption by the mice.

The forced swim has been widely used for assessing the effectiveness of antidepressant candidates. In line with earlier findings,[8,18] in this study, the 3-week CORT injections dramatically increased the immobility time of the mice in the forced swim test, indicating behavioral despair in these animals. Treatment with honokiol (20 mg/kg) significantly reversed the CORT-induced increase in the immobility time in mice. Taken together, the results obtained from the behavioral studies indicate that honokiol treatment produced an antidepressant-like action in the CORT-treated mice.

Dysregulated HPA associated increase in plasma/serum CORT is an indicator of ongoing depression physiology.[4] Consistent with previous studies,[8,18] in the present study also CORT injections to the mice resulted in a significant increase in the serum CORT levels, which were decreased by treatment with honokiol and standard drug fluoxetine at 20 mg/kg and 15 mg/kg respectively.

BDNF is one of the most common neurotrophic factors in the central nervous system. The role of BDNF in the pathogenesis of depression and in the mechanism of action of antidepressants is well appreciated. Clinical studies have found decreased BDNF levels in the blood of depressive patients,[23,24] whereas antidepressant treatment seems able to normalize the BDNF levels.[25] Furthermore, BDNF might play an important role in CORT induced depression.[7] Several rodent studies have reported that treatment with exogenous CORT causes a significant decrease in BDNF expression in the brain regions which are critically involved in the regulation of emotion, motivation, learning and memory.[17] Consistent with these findings, in the present study, the 3-week CORT injections significantly decreased BDNF protein levels in the hippocampus of the mice, whereas honokiol treatment reversed the CORT-induced changes in BDNF expression.

5. Conclusion

In conclusion, the current study demonstrates honokiol produces an antidepressant-like effect in CORT-treated mice, which is may be mediated by increasing BDNF expression in the hippocampus.

Conflicts of interest

All authors have none to declare.

References

1. Schechter LE, Ring RH, Beyer CE, et al. Innovative approaches for the development of antidepressant drugs: current and future strategies. *NeuroRx*. 2005;2:590–611.
2. Spitzer RL, Gibbon ME, Skodol AE, Williams JBW, First MB. *DSM-IV Casebook: a Learning Companion to the Diagnostic and Statistical Manual of Mental Disorders*. 4th ed. American Psychiatric Association; 1994.
3. Mathers C, Fat DM, Boerma JT. *The Global Burden of Disease: 2004 Update*. World Health Organization; 2008.
4. Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. *Trends Neurosci*. 2008;31:464–468.
5. Zunszain PA, Anacker C, Cattaneo A, Carvalho LA, Pariante CM. Glucocorticoids, cytokines and brain abnormalities in depression. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011;35:722–729.
6. Johnson SA, Fournier NM, Kalynchuk LE. Effect of different doses of corticosterone on depression-like behavior and HPA axis responses to a novel stressor. *Behav Brain Res*. 2006;168:280–288.
7. Dwivedi Y, Rizavi HS, Pandey GN. Antidepressants reverse corticosterone-mediated decrease in brain-derived neurotrophic factor expression: differential regulation of specific exons by antidepressants and corticosterone. *Neuroscience*. 2006;139:1017–1029.
8. Zhao Y, Ma R, Shen J, Su H, Xing D, Du L. A mouse model of depression induced by repeated corticosterone injections. *Eur J Pharmacol*. 2008;581:113–120.
9. Gregus A, Wintink AJ, Davis AC, Kalynchuk LE. Effect of repeated corticosterone injections and restraint stress on anxiety and depression-like behavior in male rats. *Behav Brain Res*. 2005;156:105–114.
10. Shen J, Liang Z. Advances in research on biological etiology of depression and the antidepressants. *Pharm Care Res*. 2007;7:94.
11. Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT. Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. *PLoS Med*. 2008;5:e45.
12. Maruyama Y, Kuribara H. Overview of the pharmacological features of honokiol. *CNS Drug Rev*. 2000;6:35–44.
13. Liu H, Zang C, Emde A, et al. Anti-tumor effect of honokiol alone and in combination with other anti-cancer agents in breast cancer. *Eur J Pharmacol*. 2008;591:43.
14. Xu Q, Yi L-T, Pan Y, et al. Antidepressant-like effects of the mixture of honokiol and magnolol from the barks of *Magnolia officinalis* in stressed rodents. *Prog Neuropsychopharmacol Biol Psychiatry*. 2008;32:715–725.
15. Huston JP, Schulz D. Toward an animal model of extinction-induced despair: focus on aging and physiological indices. *J Neural Transm*. 2009;116:1029–1036.
16. Ibarguen-Vargas Y, Surget A, Vourc'h P, et al. Deficit in BDNF does not increase vulnerability to stress but dampens antidepressant-like effects in the unpredictable chronic mild stress. *Behav Brain Res*. 2009;202:245–251.
17. Huang Z, Zhong X-M, Li Z-Y, Feng C-R, Pan A-J, Mao Q-Q. Curcumin reverses corticosterone-induced depressive-like behavior and decrease in brain BDNF levels in rats. *Neurosci Lett*. 2011;493:145–148.
18. Wu T-C, Chen H-T, Chang H-Y, et al. Mineralocorticoid receptor antagonist spironolactone prevents chronic corticosterone induced depression-like behavior. *Psychoneuroendocrinology*. 2012.
19. Kuribara H, Kishi E, Kimura M, Weintraub ST, Maruyama Y. Comparative assessment of the anxiolytic-like activities of honokiol and derivatives. *Pharmacol Biochem Behav*. 2000;67:597–601.
20. Mao Q-Q, Ip S-P, Ko K-M, Tsai S-H, Che C-T. Peony glycosides produce antidepressant-like action in mice exposed to chronic unpredictable mild stress: effects on hypothalamic–pituitary–adrenal function and brain-derived neurotrophic factor. *Prog Neuropsychopharmacol Biol Psychiatry*. 2009;33:1211–1216.
21. Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther*. 1977;229:327.

22. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. *J Pharmacol Pharmacother*. 2010;1:87.
23. Brunoni AR, Lopes M, Fregni F. A systematic review and meta-analysis of clinical studies on major depression and BDNF levels: implications for the role of neuroplasticity in depression. *Int J Neuropsychopharmacol*. 2008;11: 1169.
24. Karege Fl, Perret G, Bondolfi G, Schwald Ml, Bertschy G, Aubry J-M. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res*. 2002;109:143–148.
25. Shimizu E, Hashimoto K, Okamura N, et al. Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry*. 2003;54:70–75.

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Original article

Adaptogenic activity of lanostane triterpenoid isolated from *Carissa carandas* fruit against physically and chemically challenged experimental mice

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ABSTRACT

Objective: *Carissa carandas* L. an important plant bearing minor fruit has been traditionally, used for the aphrodisiac, aperitive, antipyretic and astringent properties. Lanostane triterpenoid isolated from the ethanolic fruit extract and investigated for adaptogenic activity in experimental mice.
Methods: The lanostane triterpenoid was isolated from the ethanolic extract of the fruit and characterized as Lanost-5-en-3β-ol-21-oic acid on the basis of spectroscopic (UV, IR, ^{1}H, ^{13}C NMR and MS) methods. Ethanolic extract at a dose of 100 and 200 mg/kg/day as well as lanostane triterpenoid (10 mg/kg/day) were screened for adaptogenic activity using anoxia stress tolerance, swimming endurance and Cyclophosphamide induced immunosuppression model. The levels of Hb, RBC, WBC and organ and body weight suppressed by Cyclophosphamide were estimated.
Results: The isolated compound was amorphous brownish white powder yielded 0.44% (w/w), melting point 130–132 °C, R_f value at 0.81 in methanol:chloroform (90:10) solvent system, UV absorption maxima at 283 nm and molecular peak $[M + H]^+$ at 458.4 *m/z*. It was observed that extract and lanostane triterpenoid significantly increased the anoxia stress tolerance, swimming endurance, duration of stay on rota rod and normalized the Hb, RBC, WBC level, altered organ and body weight ($P < 0.05$ and $P < 0.01$) suppressed by Cyclophosphamide.
Conclusion: These findings demonstrate that extract and isolated compound showed significant adaptogenic activity. Mass spectra and ^{13}C NMR confirmed the isolated compound structure which was supported by ^{1}H NMR.

1. Introduction

Everyone wants to look smart, maintain his body charming and powerful life for long time. But in this stressful environment it is very difficult to maintain body youth. Because in stressful environment the energy requirement of organisms are increased resulting in enhance generation of free radicals causes oxidation of lipid, proteins and nucleic acids.[1] During this process, the ability of the body's defence system to combat the oxidative stress may be diminish.[2] Natural product resources provide excellent raw material for the discovery and development of novel oxidative stress defence and anti-ageing compounds.[3] Terpenoid, steroid, organic acids, flavonoids and phenolic glycoside have been reported to exhibit a wide range of biological activity and this effect is mainly attributed to their possessing antioxidant, mast cells stabilizing and adaptogenic properties.[4]

Carissa carandas L. (Apocynaceae), an important plant bearing minor fruit is commonly known as Karonda (Christ's thorn) and has been cultivated in a limited way in the tropical, subtropical and mediterranean region.[5] Traditionally, the sour unripe fruit is reputed for the aphrodisiac, aperitive, antipyretic and astringent properties used in the treatment of diarrhoea and intermittent fever.[6] The ripe fruit is acidic and cooling used to treat mouth ulcer, sore throat and skin disorders and is the rich source of iron (39.1 mg/100 g) and possesses appreciable amount of jelly grade pectin hence a large number of processing factories have been built for making commercial jam/jelly and a product by the name 'Nakal cherry' which closely resembles the canned cherry fruits.[7] The chemical investigations of selected plant had led to the isolation of several chemical entities including β-sitosterol, lupeol, and mixture of cardenolides carissone and carindone.[8] Although, the plant

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possesses several pharmacological actions and potential therapeutic applications, no scientific claim has been made on adaptogenic activity. So, an attempt has been made to evaluate the adaptogenic activity of the ethanolic extract of fruit *C. carandas* (EEFCC) and lanost-5-en-3β-ol-21-oic acid (Cc-01), a major lanostane triterpenoid compound isolated from the EEFCC.

2. Materials and methods

2.1. General experimental procedure

UV spectra scanned in methanol on Lambda Bio 20 Spectrophotometer Shimadzu-U Singapore. IR spectra were recorded in KBr pellets on a Win IR FTS 135 instrument (Biorad. USA). ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Brucker Spectrometer (Brucker, USA) in $CDCl_3$ with TMS as internal standard. The MS were measured in DART dried Helium was used for ionization mode with a JEOL-AccuTOF JMS-T100LC. Melting points were determined on a Perfit melting point apparatus. Silica gel (Qualigens, 60–120 mesh, Mumbai, India) was used for column chromatography. Silica gel G (Qualigens) was used for analytical TLC. Spots were visualized by exposure to iodine vapors, UV Lamp 254 nm and by spraying with anisaldehyde sulphuric acid reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma–Aldrich Co MO, USA. Geriforte tablets (Himalaya drugs) and Cyclophosphamide were obtained from Khandelwal laboratories, Mumbai, India. The solvents for isolation were obtained from Merck Mumbai, India.

2.2. Plant material

The fruits were procured in the month of August 2010 from local market of Lucknow, UP, India and authenticated by Dr. Tarique Husain Botanist, at National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh (India). A voucher specimen No-95395 was retained in this laboratory for further reference.

2.3. Extraction and isolation

The fruits were dried in shade under hot air blower and powdered. The fruit powder (500 g) was defatted with petroleum ether and extracted with ethanol by using Soxhlet apparatus. The ethanolic extract was then concentrated and dried to yield 26.43% w/w. The ethanolic extract (40 g) was submitted to Si-gel CC eluting petroleum ether, chloroform and methanol to afford 41 fractions. The compound was isolated from fractions 11–17 (chloroform:methanol 90:05) and subjected to TLC using the solvent system chloroform:methanol (90:10) which showed a single spot at R_f value of 0.81 detected by spraying with anisaldehyde sulphuric acid. This fraction was collected and dried to get 700 mg of amorphous brownish white powder with a melting point of 130–132 °C which was coded as Cc-01.

2.4. Animals

Healthy adult Swiss Albino mice of either sex weighing between 20 and 25 g were procured from Central Drug Research Institute Lucknow, India. They were maintained in clean, sterile, polypropylene cages at room temperature (21 ± 2 °C) in 12 h dark/light control and fed with commercial pellet and water *ad libitum*. After randomization into various groups, the mice were quarantined for the period of a week for environmental and trainer handling acclimatization before initiation of experiment like anoxia stress tolerance test, swimming endurance test and Cyclophosphamide induced immunomodulator test. The experimental protocol was approved by the Institutional Ethical Committee (Approval no – 1213/ac/08/CPCSEA/IU) following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) which complies with International norms of INSA (Indian National Science Academy).

2.5. Administration of drugs

The ethanolic extract of fruit *C. carandas* (EEFCC) its isolated constituent Cc-01 was used in all the experimental models. Geriforte tablets (Himalaya drugs) 50 mg/kg was used as a standard adaptogenic drug which is a multi-constituent ayurvedic drug with 35 herbal and natural constituents like *Withania somnifera*, *Asparagus racemosus*, *Glycyrrhiza glabra*, *Centella aciatica*, *Terminalia chebula*, *Piper longum*, Shilajit etc.[9] All the drugs were formulated into an emulsion using gum acacia (2%) to obtain the desired dose on body weight basis (mg/kg) of the animal and administered orally using a ball ended feeding needle.

2.6. Safety profile study

An acute toxicity study of the ethanolic extract of fruit *C. carandas* (EEFCC) and isolated compound Cc-01 were carried out for the determination of LD_{50} by adopting the fixed dose method (Annexure 2d) of CPCSEA, OECD guideline no. 420.[10] The number of dead or surviving mice after 24 h was recorded.

2.7. Anoxia stress tolerance

Thirty Swiss Albino mice were divided in to 5 groups of 6 mice in each ($n = 6$) for study. Group-I served as control and receive vehicle alone 5 ml/kg/day (2% gum acacia). Group-II and -III were treated with 100 and 200 mg/kg/day EEFCC. Group-IV was treated with 10 mg/kg Cc-01 and Group-V was treated with standard drug Geriforte 50 mg/kg/day for 3 weeks respectively. Every week after 1 h of drug administration, each animal was placed in an air-tight 250 ml glass container and the time taken for the development of clonic convulsion was taken as the end point.[11] The time duration from the entry of the animal into the hermetic vessel and the appearance of first convulsion was taken as time of anoxia tolerance.

2.8. Swimming endurance and post-swimming motor function test

Mice were divided into five groups of six mice each ($n = 6$). Mice of the Group-I served as control and received vehicle alone (5 ml/kg/day 2% gum acacia). Group-II and -III were treated with 100 and 200 mg/kg/day of EEFCC respectively. Group-IV was treated with 10 mg/kg/day Cc-01 and Group-V was treated with standard drug (Geriforte 50 mg/kg/day). All the drugs were given orally once a day for seven days. On seventh day 1 h after drug administration all the mice were made to swim in a water tank (140 × 60 × 45 cm) maintained at room temperature until they sank. This was recorded as the swimming time.[12] Mice were removed and allowed to recover for about 5 min. They were subsequently tested for muscle coordination on a rota rod (Medicraft rota rod M. no.519/E-30, India) rotating at 15 rpm and the duration of stay on the rod was recorded.[13]

2.9. Cyclophosphamide (Cyp) induced immunosuppression

In this experiment, Cyclophosphamide (Cyp) was used as to suppress the immune activity of mice.[14] Mice were divided into four groups containing six mice in each group. Group-I, the control group received (5 ml/kg/day 2% gum acacia) while Group-II received Cyp 25 mg/kg/day alone and Group-III Cyp 25 mg/kg/day

along with 100 mg/kg/day EEFCC and Group-IV received 10 mg/kg/day Cc-01 along with Cyp 25 mg/kg/day for 16 days. At the end of the treatment schedule, mice were anaesthetized and blood sample was collected by cardiac puncture into tubes containing Ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant.[15] For hematological studies blood was collected. The vital organs (liver, kidney, spleen, and heart) were carefully dissected out, cleaned of the adhering connective tissues, blotted, accurately weighed and relative organ weight was calculated. The WBC count was done by Turke's method,[16] RBC by Hayem's method[17] and haemoglobin by Sahli's method.[18] The body weight of animal was also recorded prior to Cyp treatment and every 4th day upto 16 days period.

2.10. Statistical analysis

The data was represented as mean ± S.E.M. for six mice. One-way analysis of variance (ANOVA) followed by Dunnett's test was performed by using Graph Pad Prism 2.01 (Graph Pad Software Inc.). The data were expressed as the mean ± standard error of the means (SEM) and a value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Structure elucidation of lanost-5-en-3β-ol-21-oic acid (Cc-01)

Amorphous brownish white powder; yield 0.44% (w/w); mp 130–132 °C; R_f: 0.81 (MeOH:$CHCl_3$, 90:10); UV–visible λ_{max} nm ($CHCl_3$): 283; IR υ_{max} (KBr): OH (str) 3450; Ali C–H (str) 2924; C=C 1637; C=O 1701; OH (bend) 1216; C–C (str) 929 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$) spectral data: Table 1; MS DART *m/z* (rel. int.): 458.4 $[M]^+$ ($C_{30}H_{50}O_3$), 440.39, 412.39, 248.18, 207.18 and 191.18.

Table 1
^1H and ^{13}C NMR spectral values of Lanostenoloic acid (Cc-01).

Position of proton	Nature of proton	δ_H (ppm)		δ_C (ppm)
		α	β	
C_1	CH_2	1.25 (*brs*)	1.29	37.73
C_2	CH_2	1.43 (*brs*)	1.50	27.68
C_3	CH	3.21 (*dd*, $J = 5.2$)	–	66.17
C_3	OH	5.01	–	–
C_4	C	–	–	38.45
C_5	C	–	–	138.01
C_6	CH	5.28 (*d*, $J = 9.99$)		123.16
C_7	CH_2	2.27	2.31 (*m*)	29.17
C_8	CH	2.34 (*d*, $J = 6.0$)		41.88
C_9	CH	2.13		48.28
C_{10}	C	–	–	38.24
C_{11}	CH_2	2.17	2.20	21.60
C_{12}	CH_2	1.98	2.02	35.85
C_{13}	C	–		44.23
C_{14}	C	–		54.96
C_{15}	CH_2	1.87 (*brs*)	1.90	34.27
C_{16}	CH_2	1.62 (*brs*)	1.68	30.11
C_{17}	CH	1.81 (*m*)		50.72
C_{18}	CH_3	0.85 (*brs*)		18.02
C_{19}	CH_3	0.90 (*m*)		21.03
C_{20}	CH	2.49 (*d*, $J = 5.7$)		40.42
C_{21}	C	–		182.15
C_{22}	CH_2	1.17		36.15
C_{23}	CH_2	1.13	1.08	22.68
C_{24}	CH_2	1.03		45.16
C_{25}	CH	1.54 (*d*)		33.52
C_{26}	CH_3	0.78		22.15
C_{27}	CH_3	0.77		22.08
C_{28}	CH_3	0.95 (*d*, $J = 7.8$)		22.21
C_{29}	CH_3	0.93 (*d*, $J = 7.8$)		22.03
C_{30}	CH_3	0.88 (*brs*)		16.33

Coupling constants in Hz are provided in parentheses.

Table 2
Effect of ethanolic extract of fruit *C. carandas* (EEFCC) on anoxia stress tolerance test in mice.

Treatment groups	Mean duration of anoxia tolerance time in mice (min)			
	0 Week	1st Week	2nd Week	3rd Week
Control vehicle 5 ml/kg	38.12 ± 1.625	40.22 ± 1.855	43.16 ± 1.749	44.24 ± 1.806
EEFCC 100 mg/kg	40.16 ± 1.522	46.18 ± 1.285^{ns}	49.24 ± 1.213*	51.21 ± 1.121**
EEFCC 200 mg/kg	39.18 ± 1.632	48.16 ± 2.064*	51.14 ± 1.600**	54.08 ± 2.154**
Cc-01 10 mg/kg	40.14 ± 1.642	45.14 ± 1.184	52.16 ± 1.785**	56.15 ± 1.652**
Geriforte 50 mg/kg	39.16 ± 1.728	50.20 ± 2.118**	56.12 ± 1.114**	59.17 ± 2.811**

Values are expressed as Mean ± S.E.M. of six mice in each group. One-way (ANOVA) analysis of variance was performed followed by Dunnett's test.
*$p < 0.05$ & **$p < 0.01$ compare to respective control group.

3.2. Safety profile study

Acute toxicity studies of the ethanolic extract of *C. carandas* did not exhibit any signs of toxicity up to 2 g/kg body weight. Since there was no mortality of the animals found at highest dose, hence, 100 and 200 mg/kg doses of the extract was selected for evaluation of adaptogenic activity.

3.3. Anoxia stress tolerance

It was observed that EEFCC, Cc-01 and standard drug (Gerifort) significantly enhanced the anoxia tolerance time ($P < 0.05$ and $P < 0.01$). The anoxia tolerance effect was increased with dose and duration of treatment (Table 2). Pretreatment with EEFCC and Cc-01 observed that increase in anoxia stress tolerance time indicating the significant adaptogenic activity.

3.4. Effect on swimming endurance test and post-swimming motor function test

Fig. 2 shows the swimming capabilities of mice treated with EEFCC, Cc-01 and Geriforte over control group of mice. The control group of mice swam for 131.2 ± 4.934 min, EEFCC treated mice at a dose of 100 and 200 mg/kg/day swam for 148.3 ± 5.784 and 154.8 ± 3.916 min whereas Cc-01 treated mice swam for 159.6 ± 4.864 min at a dose of 10 mg/kg. Fig. 3 shows that the duration of stay on rota rod which was significantly increased from

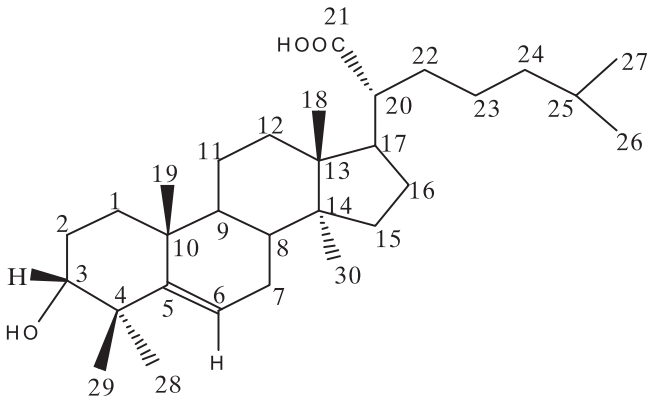

Fig. 1. Structures of the isolated compounds Lanost-5-en-3β-ol-21-oic acid (Cc-01).

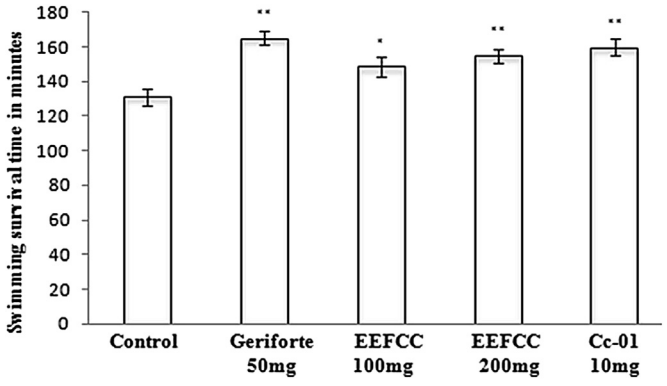

Fig. 2. Effect of ethanolic extract of fruit *C. carandas* (EEFCC) on swimming endurance test in mice. Values are represented as Mean ± SEM ($n = 6$). $^{*}p < 0.05$ and $^{**}p < 0.01$ were compare with respective control group.

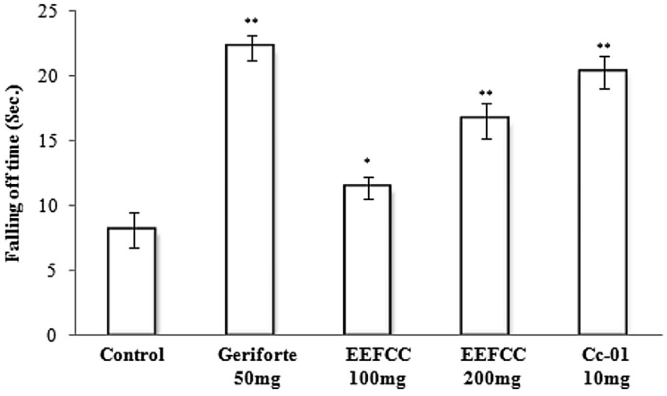

Fig. 3. Effect of ethanolic extract of fruit *C. carandas* (EEFCC) on post-swimming motor function in mice. Values are represented as Mean ± SEM ($n = 6$). $^{*}p < 0.05$ and $^{**}p < 0.01$ were compare with respective control group.

8.12 ± 1.340 s in control group to 11.4 ± 0.862 s and 16.6 ± 1.371 s in the group treated with EEFCC at dose of 100 and 200 mg/kg/day, whereas 20.32 ± 1.210 s in the group treated with Cc-01 at 10 mg/kg/day.

3.5. Cyclophosphamide induced immunosuppression

Administration of Cyp (25 mg/kg/day) alone produced a significant decrease in the total RBC count from 6.91 ± 0.096 to 5.12 ± 0.081, leukocyte count from 9.36 ± 0.156 to 3.26 ± 0.115, haemoglobin from 16.40 ± 0.071 to 14.35 ± 0.086 and total body weight from 22.79 ± 0.86 to17.27 ± 0.83. Cyp given along with EEFCC (100 mg/kg/day) and Cc-01 (10 mg/kg/day) indicated good protection by significantly increasing all the haematological parameters and maintain the organ and body weight (Table 3). Mice treated with Cyp alone were also observed that a decrease in the liver, spleen and heart weight and their treatment with Cyp along with EEFCC and Cc-01 showed significantly ($P < 0.05$ and $P < 0.01$) maintain the weight of liver, spleen and heart as compare to Cyp alone treated group (Table 4).

4. Discussion

On the basis of ^{1}H and ^{13}C NMR data the structure of the compound, Cc-01 is shown in (Fig. 1). The compound Cc-01 was obtained as amorphous brownish white powder. It gave positive Salkowiski test for triterpenoid and showed IR absorption band for hydroxyl groups (3450, 3260 cm^{-1}), unconjugated C=C group (1613 cm^{-1}) and aliphatic chain (929 cm^{-1}). On the basis of mass and ^{13}C NMR spectra the molecular peak of Cc-01 was found m/z 458.39 $[M]^{+}$ consistent to the molecular formula $C_{30}H_{50}O_3$. It may be due to loss of CO_2 molecule during the ionization of the compound in position C_{21} the molecular ions found in the mass spectra were corresponding the mass peak at m/z 412. The compound is fragmented in two parts between C_{11} and C_{12} and between C_8 and C_{14}. These fragments have m/z 191 and 248 with molecular formula $C_{14}H_{22}$ and $C_{18}H_{32}$ respectively. The ^{1}H NMR spectrum of the Cc-01 showed a series of proton signal (1.25–1.90 ppm) attributed to resonance of overlapping of methylenes and methines a characteristic framework of triterpenoid. The double doublet signal at 5.28 ppm indicates the presence of ethylenic proton at C-6 and coupled with H-7a and H-7b. The methynic proton which is in a deshielded (CH) environment gives a double doublet signal at 3.21 and another methynic proton gives singlet peak at 1.50 ppm. The gem dimethyl protons (CH_3) give a broad multiplete signal at 0.88 ppm. The hydroxy proton (−OH) is identified with the singlet signal at 5.0 ppm. The spectrum further displaced signal at 0.78 ppm (H_{18}), 1.08 ppm (H_{19}) and 0.90 ppm (H_{26} and H_{27}) characteristic of 5-ene-3-β-hydroxy terpenoids. The ^{13}C NMR showed peaks at δ_C 18.02, δ_C 21.03, δ_C 182.15 corresponding to C_{18}, C_{19} and C_{21}, respectively. The C_3 and C_{23} resonated at δ_C 66.17 and 22.68, respectively. C_5 resonated at δ_C 138.01 while C_6 resonated at δ_C 123.16. On the basis of these evidences the structure of Cc-01 has been established as Lanost-5-en-3β-ol-21-oic acid.[19]

Modern life style has enhanced the exposure of human beings to stressful conditions resulting in the physical, psychological abnormalities. Therefore, there is a need to enhance the adaptability of human beings to stressful conditions.[11] In the present study an attempt was made to evaluate the adaptogenic property of traditionally used fruit *C. carandas*. Anoxia stress, swimming endurance test and post-swimming motor function test and Cyclophosphamide induced immunosuppression models were used for evaluation of adaptogenic activity. The study on ethanolic extracts of *C. carandas* fruit and isolated compound lanostane triterpenoid showed significant adaptogenic activity against in all selected model.

Anoxia is a very severe form of stress. All the body functions including cellular respiration depend on oxygen supply to them.

Table 3
Effect of ethanolic extract of fruit *C. carandas* (EEFCC) on Cyclophosphamide (Cyp) induced changes in body weight, hemoglobin (Hb), RBC and WBC count.

Treatment group	Changes in body weight	Hb (gm%)	RBC count million/cumm.	WBC count cells/10^3/cumm.
Control vehicle 5 ml/kg	22.79 ± 0.86	16.40 ± 0.071	6.91 ± 0.096	9.36 ± 0.156
Cyp 25 mg/kg	17.27 ± 0.83	13.35 ± 0.086*	4.12 ± 0.081*	3.26 ± 0.115**
Cyp + EEFCC 100 mg/kg	17.72 ± 0.84	15.18 ± 0.087$^{\#}$	5.71 ± 0.197$^{\#}$	7.14 ± 0.154$^{\#\#}$
Cyp + Cc-01 10 mg/kg	21.76 ± 0.77	16.18 ± 0.126$^{\#\#}$	6.35 ± 0.089$^{\#\#}$	8.87 ± 0.168$^{\#\#}$

Values are expressed as Mean ± S.E.M. of six mice in each group. One-way (ANOVA) analysis of variance was performed followed by Dunnett's test.
$^{*}p < 0.05$ & $^{**}p < 0.01$ compare to respective control group.
$^{\#}p < 0.05$ & $^{\#\#}p < 0.01$ compare with Cyclophosphamide (Cyp) treated group.

Table 4
Effect of ethanolic extract of fruit *C. carandas* (EEFCC) on Cyclophosphamide (Cyp) induced changes on the relative organ weight of mice.

Treatment group	Liver ($\times 10^{-3}$)	Spleen ($\times 10^{-3}$)	Kidney ($\times 10^{-3}$)	Heart ($\times 10^{-3}$)
Control vehicle 5 ml/kg	14.14 ± 0.05	1.19 ± 0.09	4.14 ± 0.11	7.15 ± 0.09
Cyp 25 mg/kg	12.17 ± 0.08*	1.17 ± 0.16**	3.16 ± 0.15	5.21 ± 0.14**
Cyp + EEFCC 100 mg/kg	13.12 ± 0.09#	1.26 ± 0.12##	4.21 ± 0.12	6.18 ± 0.09##
Cyp + Cc-01 10 mg/kg	14.16 ± 0.07##	1.14 ± 0.10##	4.15 ± 0.14	7.16 ± 0.11##

Values are expressed as Mean ± S.E.M. of six mice in each group. One-way (ANOVA) analysis of variance was performed followed by Dunnett's test.
*$p < 0.05$ & **$p < 0.01$ compare to respective control group.
#$p < 0.05$ & ##$p < 0.01$ compare with Cyclophosphamide (Cyp) treated group.

Any lack of this vital element (as in anoxia) will play havoc on all body mechanisms and increase in adaptation during this stress by a drug could be considered as its major anti-stress effect. Pretreatment with ethanolic extract of *C. carandas* fruit and isolated compound observed that dose related increase in anoxia stress tolerance time indicating that the plant of the present study possesses significant adaptogenic activity. It is also suggested that adaptogenic agents facilitate the conversion of energy in cellular system of the organism and helps in adaptation.[11] Hence, during the investigation we found that EEFCC and Cc-01 facilitated conversion of energy in cellular system of organisms which could help adaptive process during anoxia tolerance stress.

The swimming endurance test and post-swimming motor function results indicate clearly that the pretreatment with EEFCC and Cc-01 have the properties whereby they significantly increase the physical swimming endurance time as well as stay on rota rod in mice. The enhanced swimming endurance in mice compared to the normal animals may be attributed to the flavonoids, acids glycosides and triterpenoids which were identified in phytochemical tests.[20]

The results demonstrate that treatment with EEFCC and Cc-01 were able to reverse the Hb, RBC, WBC, and organ and body weight significantly ($P < 0.05$ and $P < 0.01$) and they are able to reduce leukopenia and anemia induced by Cyp. No significant difference was observed in the kidneys weight after administration of Cyp alone or with test drugs. The alterations in haematological parameters, organ and body weight are usually observed in mice indicative of Cyp toxicity.[21]

5. Conclusion

The present study suggested that ethanolic extract of fruit *C. carandas* and isolated compound as lanostane triterpenoid (Cc-01) showed significant adaptogenic and immunoprotective activity. To the best of our knowledge there is no scientific report on the presence of this lanostane triterpenoid from ethanolic extract of *C. carandas* fruit through several triterpenoids and triterpenoidal glycosides. The study also affirms that the effectiveness of ethanolic extract of fruit *C. carandas* (EEFCC) is due to the presence of lanostane triterpenoid (Cc-01) which is useful marker for *Carissa* genus.

Conflicts of interest

All authors have none to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phcgj.2013.08.002.

References

1. Bhaumik G, Srivastava KK, Selvamurthy W, Purkayastha SS. The role of free radicals in cold injuries. *Int J Biometeorol*. 1995;38:171–175.
2. Kondo H, Miura M, Itokawa Y. Oxidative stress in skeletal muscle atrophied by immobilization. *Acta Physiol Scand*. 1991;142:527–528.
3. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. The relative antioxidant activities of plant derived polyphenolic flavonoids. *Free Radic Res*. 1995;22:375–383.
4. Yildirim A, Mavi A, Oktay M, Kara AA, Algur OF, Bilaloglu V. Comparison of antioxidant and antimicrobial activities of *Tilia argentea* (Desf Ex DC), *Salvia triloba* (L.) and *Camellia sinensis* extracts. *J Agric Food Chem*. 2000;48:5030–5034.
5. Bankar GJ, Verma SK, Prasad RN. Fruit for the arid region: Karonda. *Indian Hortic*. 1994;39:46–47.
6. Jayaweera DMN. *Medicinal Plant Used in Ceylon*. The National Science Council of Sri Lanka; 1981:95.
7. Mandal U, Sinha RS, Mazumdar BC. A recently developed agro industry in the southern suburb of Calcutta city, utilizing a bramble fruit. *Indian J Landscape Syst Ecol Stud*. 1992;15:100–102.
8. Singh B, Rastogi RP. The structure of carindone. *Phytochemistry*. 1972;11:1780–1797.
9. Kannur DM, Hukkeri VI, Akki KS. Adaptogenic activity of *Caesalpinia bonduc* seed extracts in rats. *J Ethnopharmacology*. 2006;108:327–331.
10. Parasuraman S. Toxicological screening. *J Pharmacol Pharmacother*. 2011;2:74–79.
11. Shivakumar H, Javed T, Prakash T, Nagendra Rao R, Jayakumar Swamy BHM, Veerana Goud A. Adaptogenic activity of ethanolic extract of *Tribulus terrestris* L. *J Nat Remedies*. 2006;6:87–95.
12. Takagi K, Saito H, Nabata H. Pharmacological studies of *Panax ginseng* root: pharmacological properties of crude saponin fraction. *Jpn J Pharmacol*. 1972;22:339–346.
13. Gufran A, Yusuf KM, Naeem A, Khan T. The anti-stress activity of a gem-containing Unani formulation against diverse stressors. *J Ethnopharmacology*. 1998;59:187–193.
14. Davis S, Kuttan G. Suppressive effect of Cyp induced toxicity by *Withania somnifera* extract in mice. *J Ethnopharmacology*. 1998;62:209.
15. Hoff J. Methods of blood collection in the mouse. *Lab Anim (NY)*. 2000;29:47–53.
16. Dacie JV, Lewis SM. *Practical Haematology*. 11th ed. Hong Kong: Longman Group. Ltd.; 2001:11–17.
17. Easthan RD, Slade RR. *Clinical Haematology*. 7th ed. Tokyo: Butter Work-Heinemann Ltd.; 1993:83–105.
18. Cavill IJ, Fisher JA, Souza P. *Clinical Lab Haematology*. 1993;3:91–93.
19. Markham KR, Ternai B, Stanley R, Geiger H, Mabry TJ. Carbon-13 NMR studies of flavonoids. Naturally occurring flavonoid glycosides and their acylated derivatives. *Tetrahedron*. 1978;34:1389–1397.
20. Klein R. Phytoecdysteroids. *J Am Herbalists Guild*. 2004;5:18–28.
21. Chahoud I, Ligensa A, Dietzel L, Faqui AS. Correlation between maternal toxicity and embryo/fetal effects. *Reprod Toxicol*. 1999;13:375.

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Original article

Antihyperlipidemic effect of Angiosifa, a polyherbal formulation, in Sprague–Dawley rats

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ABSTRACT

Objective: To study the antihyperlipidemic effect of Angiosifa, a polyherbal formulation (PHF), in Sprague–Dawley (SD) rats.
Methods: The rats were divided into seven groups, each having five animals: normal controls, high fat diet (HFD)-fed controls and HFD-fed animals treated with atorvastatin (10 mg/kg), petroleum ether extract of the PHF (200 and 400 mg/kg) and methanol extract of the PHF (200 and 400 mg/kg). The test and standard drugs were administered orally once daily for 28 consecutive days. During the experiment, changes in body weight were noted and alterations in biological and biochemical parameters were monitored at regular intervals.
Results: HFD-fed animals showed significant increases in body weight and total cholesterol, triglyceride and VLDL levels. They also showed a significant reduction in HDL levels compared with the control and drug treatment groups. Animals treated with atorvastatin and methanol extract of PHF showed significant reductions in total cholesterol, triglyceride and VLDL levels compared with HFD-fed animals. But there was no significant hypolipidemic effect in animals treated with petroleum ether extract of PHF, and the ether caused piloerection and led to cannibalism.
Conclusion: Methanol extract of PHF has a significant hypolipidemic effect against HFD-induced hyperlipidemia in SD rats. The petroleum ether extract of PHF did not show any significant hypolipidemic effect on HFD-fed rats.

1. Introduction

The concept of polyherbalism has been highlighted in the *Sarangdhar Samhita*, an Ayurvedic work dating back to 1300 A. D.[1] In a polyherbal formulation (PHF), a combination of herbs is used rather than a single herb. The individual constituents may not be sufficiently active to achieve the desired therapeutic effect. By acting on multiple targets at the same time, the PHF enhances the therapeutic efficacy by improving the bioavailability thereby reducing the doses required of the individual components in the formulation.[2] Besides, synergism attenuates undesirable side effects.[3] Raghavendra et al studied the combined effect of the methanol extract of *Tribulus terrestris* (whole plant) and *Annona squamosa* leaves against hyperlipidemia, and they concluded that a combination of the two herbs has better hypolipidemic activity compared with the individual effects.[4] Parasuraman et al demonstrated the superior antihyperlipidemic effect of a commercially available PHF that has extract of 10 herbs, *viz.*, *Terminalia arjuna*, *Cissus quadrangularis*, *Boerhaavia diffusa*, *Commiphora mukul*, *Phyllanthus emblica*, *Terminalia bellerica*, *Terminalia chebula*, *T. terrestris*, *Allium sativum* and *Trigonella foenumgraecum*.[5] Angiosifa, a PHF, is used in the management of coronary atherosclerosis, hypercholesteremia and ischemic heart diseases. Angiosifa contains seven herbs, *viz.*, *Cinnamomum zeylanicum, Glychyrizza glabra, Nelumbo nucifera,*

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Hibiscus rosa-sinesis, Sausurrea lappa, Spermacoce hispida and *T. arjuna*. The hypolipedimic effect of *H. rosa-sinensis*,[6] *S. hispida*,[7] *N. nucifera*,[8] *S. lappa*[9] and *T. arjuna*[10] are known, but the synergistic effect of these plants in the formulation has not been elucidated so far. Combinations of these herbs may have an enhanced hypolipidemic effect even with low dose levels of the individual herbs. Angiosifa consists of a raw herbal mixture, and the concentrations of the herbs are high for therapeutic efficacy. Extracts of individual plants or a formulation containing these may require a smaller dose and may have enhanced therapeutic efficacy. Hence the present study was planned to investigate the antihyperlipidemic effect of the petroleum ether and methanol extract of the PHF in rodents using the high fat diet (HFD)-induced hyperlipidemic model.

2. Materials and methods

2.1. Polyherbal formulation

Angiosifa (manufactured by East West Oriental Enterprise, Georgetown, Penang, Malaysia) is a PHF available in Malaysia. It is formulated using dried, powdered herbs, namely *C. zeylanicum, G. glabra, N. nucifera, H. rosa-sinesis, S. lappa, S. hispida* and *T. arjuna*.

2.2. Animals

Healthy adult male Sprague–Dawley (SD) rats, weighing 100 ± 10 g, were obtained from Happy Pets, Sungai Petani, Malaysia. The animals were housed in large, spacious polyacrylic cages at ambient temperature with a 12-h-light/12-h-dark cycle. The animals were acclimatized to laboratory conditions for a period of one week. The rats were fed with normal rodent pellets and water *ad libitum*. During the experiment, normal control animals were fed with a normal diet; the rest of the animals were fed with a high fat diet. The compositions of the normal and hyperlipidemic diets (HFD) are provided in Table 1. The study was approved by the Institute Animal Ethics Committee, and the study was conducted according to the guidelines of the Animal Research Review Panel (ARRP).

2.3. Extraction of PHF using petroleum ether and methanol

The PHF was packed into a Soxhlet extractor and extracted with petroleum ether at 50 °C over 6 h. The petroleum ether extract was concentrated to a dry mass using simple distillation, and the dry mass was stored under reduced pressure at room temperature. The same marc was successively extracted with methanol at 45 °C over 6 h. The methanol extract was also distilled, and the dry mass was preserved under reduced pressure at room temperature. The percentage yields of the petroleum ether and methanol extracts of the PHF were 2.56% w/v and 12.61% w/v, respectively. The extracts thus obtained were subjected to phytochemical analysis and pharmacological screening.

Table 1
Dietary composition of normal and hyperlipidemic/high fat diet.

Ingredients	Normal diet (units/kg)	HFD diet (units/kg)
Whole wheat	261 g	220 g
Yellow corn	245 g	220 g
Barley	150 g	110 g
Milk powder	150 g	165 g
Bone meal	24 g	44 g
Calcium chloride	9 g	11 g
Salt (NaCl)	9 g	11 g
Palm oil	150 ml	110 ml
Unsalted butter	–	110 g
Vitamin B complex (Cap. Becosules; Pfizer, India)	2 No.	2 No.

All the ingredients were mixed together and made into pellets of approximately 15 g each.

2.4. Phytochemical analysis of petroleum ether and methanol extract of PHF

Petroleum ether and methanol extract (500 mg) was dissolved in a minimal volume of its own mother solvent and used for phytochemical analysis. The Salkowski reaction, Tschugajeu test, Dragendroff's reaction, ferric chloride test and Baljet test were carried out to detect the presence of phytosterols, triterpenoids, alkaloids, flavonoids/phenolic compounds/tannins and glycosides, respectively.[11]

2.5. Acute toxicity testing

A single dose oral acute toxicity test was performed using the fixed dose method. Female SD rats were used for this study. The petroleum ether and methanol extract of the PHF was administered successively to different groups at fixed dose levels of 500, 1000 and 2000 mg/kg ($n = 3$ per dose) and observed for the next 14 days.[12]

2.6. Antihyperlipidemic effect of PHF

Thirty-five young, healthy male SD rats were divided into seven groups containing five animals each. The diet and treatment were as follows.

Group 1 (normal diet): Normal diet + drug vehicle (1 ml/kg)
Group 2 (HFD control): HFD + drug vehicle (1 ml/kg)
Group 3: HFD + atorvastatin (10 mg/kg)
Group 4: HFD + PHF (petroleum ether extract, 200 mg/kg)
Group 5: HFD + PHF (petroleum ether extract, 400 mg/kg)
Group 6: HFD + PHF (methanol extract, 200 mg/kg)
Group 7: HFD + PHF (methanol extract, 400 mg/kg)

The locomotor activities of all the animals were measured, and the animals that had abnormal locomotor activities were excluded from the study. All the animals were provided with 15 g normal/HFD diet per day. The drug vehicle, standard drug (atorvastatin) and PHF were administered once daily as an oral suspension for 28 days. The oral suspension was prepared with 0.5% w/v carboxymethyl cellulose (CMC). All the experimental procedures were carried out between 9:00 am and 11:00 am to avoid chronopharmacological variations. Throughout the study, the behavioral alterations, body weight variations and biochemical changes were monitored at regular intervals. At the end of the study, all the animals were sacrificed by cervical dislocation. The liver and kidney were excised, and the absolute organ weights were measured. A part of the liver tissue of each of the experimental animals was preserved in 10% v/v buffered neutral formalin for histopathological examination.

2.7. Measurement of locomotor activity

The activity of the rats was recorded in a rodent activity cage (actophotometer) provided with an acrylic cage and with 16 beams of infrared light along both the *x* and *y* axes. The activity of each rat was monitored at room temperature over 10 min[13]

2.8. Body weight analysis

The body weight of each rat in each group was recorded initially and at weekly intervals. The percentage change in body weight was calculated.

2.9. Collection of serum

A blood sample (0.5 ml) was collected from each experimental animal on the day preceding the commencement of the study and on the 14th and 28th days. The samples were drawn from the retro-orbital sinus under mild ether anesthesia.[14] The blood samples were collected in sodium EDTA tubes and centrifuged at 3000 RPM for 20 min. The plasma obtained was maintained at −80 °C until analysis.

2.10. Biochemical estimations

The plasma was used to estimate the plasma lipid profile. The total cholesterol, triglyceride and HDL levels were measured using a Reflotron biochemical analyzer (Reflotron Plus System, Hoffmann-La Roche, USA) and lipid profile analytical strips. The LDL, HDL ratio and atherogenic index (AI) were calculated mathematically.[5]

2.11. Histopathological study

A part of each liver sample was embedded in paraffin after being dehydrated in alcohol and subsequently cleared with xylene. Liver sections of thickness 5 μm were prepared from paraffin blocks, stained with hematoxylin and eosin and mounted in neutral DPX medium. The sections were examined under a light microscope.[15]

2.12. Statistical analysis

All the values are represented as the mean ± S.E.M. Statistical differences among the groups were determined using One-way repeated measures ANOVA, followed by the Bonferroni post hoc test. $P < 0.05$ was considered to be significant.

3. Results

3.1. Phytochemcial analysis of petroleum ether and methanol extract of PHF

The primary phytochemical analysis of the petroleum ether extract of the PHF showed the presence of phytosterols, triterpenoids, alkaloids, flavonoids, phenolic compounds and tannins, and analysis of the methanol extract of the PHF showed the presence of phytosterols, triterpenoids, flavonoids and glycosides.

3.2. Acute toxicity testing

No mortality or signs of toxicity were noted on the first day or during the study period of the single-dose oral toxicity testing of the petroleum ether and methanol extracts of PHF at dose levels of 500, 1000 and 2000 mg/kg. Hence, the present study was initiated with dose levels of 200 and 400 mg/kg. Repeated administration (antihyperlipidemic study) of the petroleum ether extract of the PHF produced signs of toxicity including altered locomotor activity, piloerection and cannibalism.

3.3. Antihyperlipidemic effect of petroleum ether and methanol extract of PHF

3.3.1. Body weight analysis

HFD-fed animals exhibited a significant increase in body weight compared with normal diet-fed animals from the third week onwards ($P < 0.01$). The effects of the PHF on the body weight over the period of 28 days of treatment were studied and recorded in all the groups. The percentage changes in body weight were calculated from the weights noted before the study and on the 7, 14, 21 and 28th days (Fig. 1). The HFD-fed animals that were administered the petroleum ether extract of the PHF showed significant increases in body weight; those administered atorvastatin and the methanol extract of the PHF did not show any significant increase in body weight compared with the control animals.

3.3.2. Locomotor activity

The HFD-fed animals showed a significant decrease in locomotor activity at the end of the study compared with the controls ($P < 0.05$). The groups treated with the PHF and with atorvastatin did not display altered locomotor activity (Table 2). There were no significant alterations in absolute organ weight in the HFD control group and the treatment groups. This may be due to the short duration of the study.

3.3.3. Effect of petroleum ether extract of PHF on central nervous system

Repeated administration of the petroleum ether extract of the PHF at 200 and 400 mg/kg caused rigidity and dorsiflexion of the rats' tails (piloerection reaction/Straub tail phenomenon) and cutis anserina. Cannibalism was noted in the group treated with the petroleum ether extract of the PHF at 200 mg/kg after the 12th day of drug administration (Table 3).

3.3.4. Intake analysis

Control animals consumed more food and water (Figs. 2 and 3) compared with the other groups. There were no significant changes in the food and water intake of the animals treated with atorvastatin or the PHF. A quantitative decrease in food intake and a significant decrease in water intake were observed with HFD-fed animals.

3.3.5. Serum lipid profile

The effects of the PHF on the biochemical parameters (total cholesterol, triglyceride, HDL, VLDL and LDL levels; HDL ratio; and AI) were assessed from measurements made before the commencement of the study and on the 14th and 28th days of treatment (Tables 4–6).

The HFD-fed rats showed significant increases in total cholesterol and triglyceride levels from the second week onwards. The petroleum ether and methanol extract of the PHF inhibited the hyperlipidemia caused by the HFD. Compared with the petroleum ether and methanol extract of the PHF, the methanol extract of the PHF showed better activity.

In the second week of the study, there were significant increases in the total cholesterol (28.98%) and triglyceride (49.87%) levels of the HFD-fed animals compared with the controls. In contrast, the atorvastatin- and PHF-treated groups did not have significant alterations in the total cholesterol and triglyceride levels compared with the controls.

At the end of the study, the HFD-fed animals had significant increases in total cholesterol (34.66%) and triglyceride (60.40%) levels compared with the controls. The atorvastatin- and PHF-treated groups did not have significant alterations in their total cholesterol and triglyceride levels compared with the controls. The

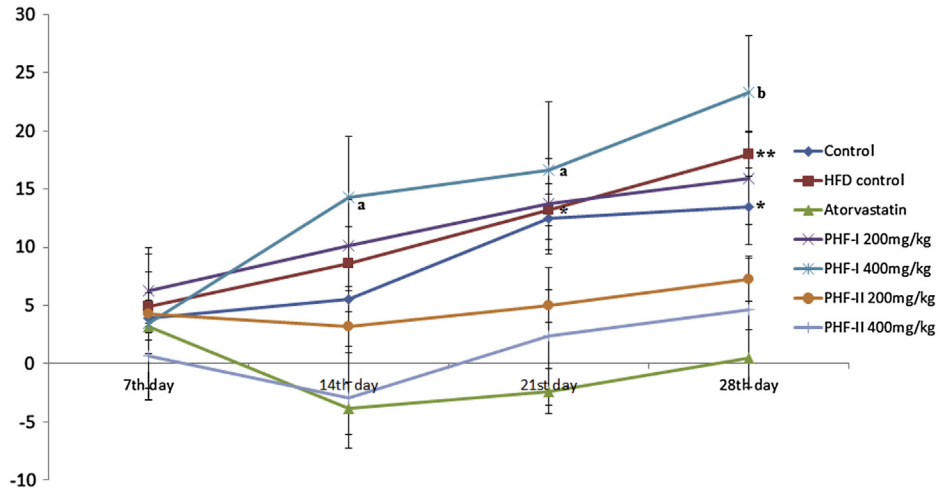

Fig. 1. Effect of petroleum ether and methanol extracts of PHF on animal growth (percentage body weight changes). PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$) except PHF-I 200 mg/kg except PHF-I 200 mg/kg (n in PHF-I is a = 3 and $b = 2$). Percentage weight changes calculated using pre-study values. $^{*}P < 0.05$, $^{**}P < 0.01$ compare to pre-study day body weight, One-way repeated measures ANOVA followed by Bonferroni post hoc test.

methanol extract of the PHF at dose levels of 200 and 400 mg/kg reduced the total cholesterol by 4.99% and 0.29%, respectively, compared with the controls.

Throughout the experiment, decreased HDL levels and increased VLDL levels were noted in the HFD-fed animals compared with the controls. The LDL and VLDL levels of atorvastatin were significantly reduced at the end of the study compared with the HFD-fed controls, and the methanol extract of the PHF (at 200 and 400 mg/kg) were significantly reduced the LDL level compared with the HFD controls. Throughout the study, atorvastatin and PHF treatment did not have any significant influence the AI values.

3.4. Histopathological analysis

The liver of HFD-fed animals had diffuse fatty infiltration affecting all the hepatocytes, leading to peripherally compressed nuclei, and minimal periportal inflammation (Plate 1). This infiltration was not seen in the controls. Normal morphological features were observed in normal control, atorvastatin and PHF treated animals liver.

4. Discussion

Phytochemical screening of the petroleum ether and methanol extract of the PHF showed the presence of phytosterols, which may contribute to the hypolipidemic effect of the PHF by reducing cholesterol absorption in the gastrointestinal tract. Cholesterol may be displaced from micelles by the phytosterols, thereby limiting the solubility of cholesterol in the small intestine.[16] Flavonoids, which are among the contents of the PHF, are generally known to display hypolipidemic activity, and they may act through inhibition of HMG CoA reductase[17] as well as increasing resistance to LDL oxidation.[18] Increased HDL levels and HDL ratios were observed in the atorvastatin and PHF-treated groups, and these effects may help modulate endothelial function.[19]

The results of the present study support the assertion that the PHF has a hypolipidemic effect. The methanol extract of the PHF significantly inhibited diet-induced hyperlipidemia in rats compared with the petroleum ether extract. The HFD control group had significant increases in the plasma lipid parameters (TG, TC, LDL and VLDL) compared with the controls. Atorvastatin and PHF treatment reversed the effects of HFD, and this effect may be due to inhibition of either the cholesterol metabolism or a reduction in food intake. Both atorvastatin and PHF do not produce any significant changes in LDL, VLDL, HDL ratio and AI. This may be due to the short duration of the study and the small sample size. If we had only conducted our study with more than eight animals in each group or increased the duration of the study to 60/90 days, we would have made a statistical difference to the LDL, VLDL, HDL ratio and AI. At the end of the study, none of the animals from the treatment groups had any changes in the absolute organ weights or histopathological changes.

Throughout the experiment, the normal control animals were found to have increased food and water intake, but HFD-fed animals had increased food intake only till the second week. Except

Table 2
Effect of petroleum ether and methanol extracts of PHF on locomotor activity.

Group	Locomotor count (10 min)
Control	534 ± 10.30
HFD control	302 ± 62.45$^{\$}$
Atorvastatin	552 ± 64.66*
PHF-I 200 mg/kg	442.5 ± 92.51[a]
PHF-I 400 mg/kg	687 ± 77.31***
PHF-II 200 mg/kg	435 ± 37.82
PHF-II 400 mg/kg	551 ± 35.01*

PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$). $^{\$}P < 0.05$ as compared to control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compare to HFD control, One-way ANOVA followed by Bonferroni post hoc test.
[a] $n = 2$.

Table 3
Effect of petroleum ether and methanol extracts of PHF on cannibalism.

Group	History of cannibalism
Control	Nil
HFD control	Nil
Atorvastatin	Nil
PHF-I 200 mg/kg	12th day of dosing – 2 animals 18th day of dosing – 1 animal
PHF-I 400 mg/kg	Nil
PHF-II 200 mg/kg	Nil
PHF-II 400 mg/kg	Nil

PHF-I: Pet-ether extract; PHF-II: Methanol extract.

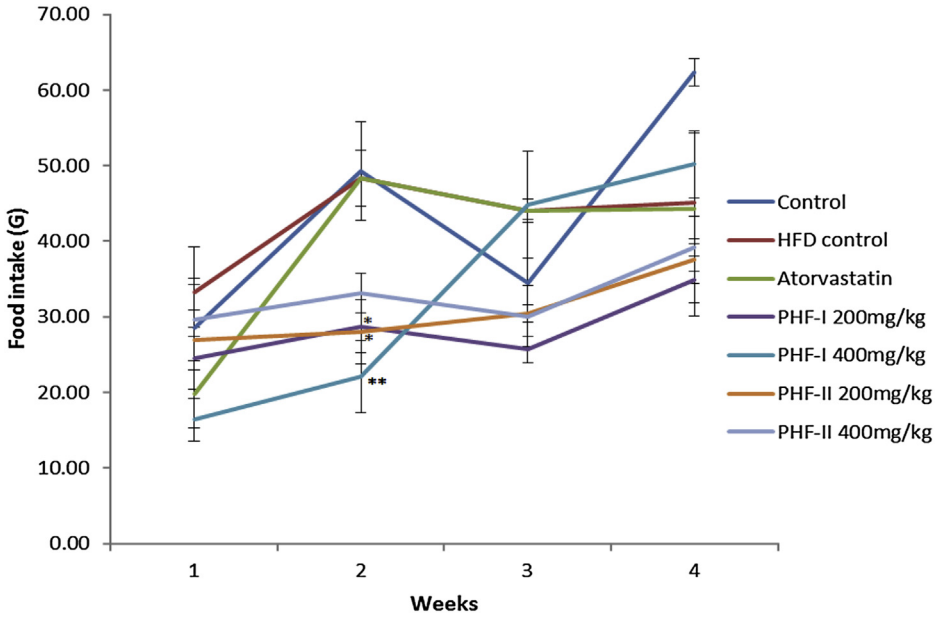

Fig. 2. Effect of petroleum ether and methanol extracts of PHF on food intake (g). PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$) except PHF-I 200 mg/kg ($n = 3$ in 3rd week and $n = 2$ in 4th week) $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to control, One-way repeated measures ANOVA followed by Bonferroni post hoc test.

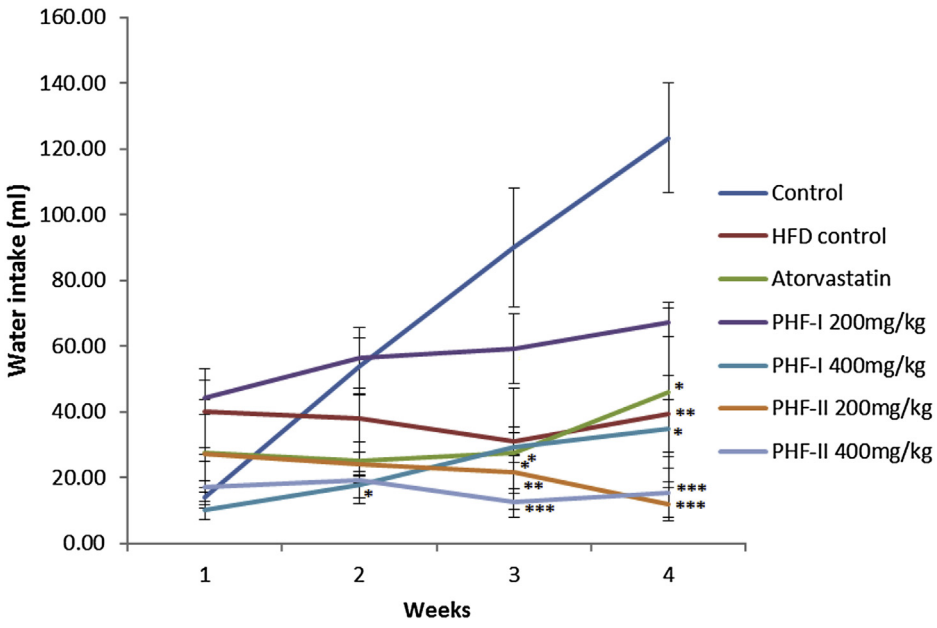

Fig. 3. Effect of petroleum ether and methanol extracts of PHF on water intake (ml). PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$) except PHF-I 200 mg/kg ($n = 3$ in 3rd week and $n = 2$ in 4th week). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to control, One-way repeated measures ANOVA followed by Bonferroni post hoc test.

Table 4
Effect of petroleum ether and methanol extracts of PHF on lipid profile in plasma (pre-study) on HFD fed SD rats.

Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	VLDL-c (mg/dl)	HDL ratio	AI
Control	106.43 ± 3.62	70.86 ± 2.80	12.65 ± 2.18	79.61 ± 3.24	14.17 ± 0.56	13.59 ± 2.43	1.14 ± 0.02
HFD control	106.19 ± 2.52	67.31 ± 5.31	13.14 ± 1.72	79.58 ± 3.13	13.46 ± 1.06	14.28 ± 2.09	1.14 ± 0.02
Atorvastatin	106.73 ± 2.45	76.17 ± 7.20	12.93 ± 1.29	78.57 ± 3.76	15.23 ± 1.44	14.05 ± 1.91	1.14 ± 0.02
PHF-I 200 mg/kg	103.71 ± 2.62	74.40 ± 9.12	14.72 ± 1.43	74.11 ± 3.58	14.88 ± 1.82	16.59 ± 1.65	1.17 ± 0.02
PHF-I 400 mg/kg	110.12 ± 1.20	76.17 ± 6.01	12.97 ± 1.90	81.92 ± 3.05	15.23 ± 1.20	13.63 ± 2.41	1.14 ± 0.02
PHF-II 200 mg/kg	106.42 ± 4.05	77.94 ± 8.12	11.46 ± 1.54	79.37 ± 3.52	15.59 ± 1.62	12.11 ± 1.62	1.12 ± 0.02
PHF-II 400 mg/kg	103.88 ± 3.50	70.86 ± 9.29	13.70 ± 2.38	76.01 ± 5.68	14.17 ± 1.86	15.89 ± 3.58	1.16 ± 0.04

PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$).

Table 5
Effect of petroleum ether and methanol extracts of PHF on lipid profile in plasma (week 2) on HFD fed SD rats.

Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	VLDL-c (mg/dl)	HDL ratio	AI
Control	109.10 ± 3.74	69.09 ± 3.32	13.06 ± 1.86	82.22 ± 2.14	13.82 ± 0.66	13.55 ± 1.78	1.14 ± 0.02
HFD control	136.97 ± 6.71**	100.97 ± 7.20*	9.45 ± 0.54	107.32 ± 6.05	20.19 ± 1.44*	7.56 ± 0.74	1.08 ± 0.01
Atorvastatin	114.44 ± 2.50	72.63 ± 9.03	23.63 ± 10.93	76.28 ± 11.09	14.53 ± 1.81	39.13 ± 26.45	1.39 ± 0.26
PHF-I 200 mg/kg[a]	118.21 ± 6.76	76.76 ± 5.90	11.18 ± 1.16	91.67 ± 4.48	15.35 ± 1.18	10.40 ± 0.54	1.10 ± 0.01
PHF-I 400 mg/kg	119.80 ± 3.95	83.26 ± 6.63	16.12 ± 5.17	87.02 ± 7.33	16.65 ± 1.33	17.57 ± 7.47	1.18 ± 0.07
PHF-II 200 mg/kg	121.66 ± 4.29	86.80 ± 8.12	16.61 ± 4.95	87.68 ± 4.65	17.36 ± 1.62	16.78 ± 6.01	1.17 ± 0.06
PHF-II 400 mg/kg	113.72 ± 1.76	70.86 ± 4.85	17.68 ± 4.21	81.86 ± 5.60	14.17 ± 0.97	19.57 ± 5.95	1.20 ± 0.06

PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$). *$P < 0.05$, **$P < 0.01$ as compared to control, One-way repeated measures ANOVA followed by Bonferroni post hoc test.
[a] $n = 3$.

the normal controls, all other experimental animals (HFD-fed animals) showed a significant reduction in food and water intake. Ghalami et al also reported a reduction in food and water intake in HFD-fed animals, and this effect might be due to increased plasma leptin levels. These increased levels may reduce protein and water intake.[20]

The petroleum ether extract of the PHF produced reductions in the TG, TC and LDL levels in rats at both a low dose (200 mg/kg) and high dose (400 mg/kg), but this effect was not comparable with that of the methanol extract of the PHF. Administration of the petroleum ether extract of the PHF resulted in Straub tail, cutis anserina, increased locomotor activity and cannibalism in the rats. This may have been due to stimulation of the central nervous system/behavioral alteration/neurological disorders or changes in environmental factors such as deficient diets and group size.[21] The effect of stimulating the central nervous system may be due to chemical changes that occurred when the PHF was extracted with petroleum ether or may be due to the solvent effect of petroleum ether. Further studies are required to study the effect of petroleum ether as a solvent on the central nervous system.

Before inducing hyperlipidemia through diet in SD rats, the authors tried to induce hyperlipidemia chemically using Triton X-100. Triton X-100 is a non-ionic surfactant, widely used in animal models to increase the concentration of lipids in the blood progressively.[22] In our experiment, Triton X-100 (100 mg/kg; i.p.) caused mortality to the extent of 60% within 2 days. Because of the high mortality rate, HFD-induced hyperlipidemia was chosen as an alternative. Sodipo et al also reported a high mortality rate with Triton X-100 (400 mg/kg; i.p.). This is probably due to high osmotic fragility and altered red blood cell (RBC) morphology, causing icterus, leading to the death of the rats.[23]

Table 6
Effect of petroleum ether and methanol extracts of PHF on lipid profile in plasma (week 4) on HFD fed SD rats.

Treatment	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)	VLDL-c (mg/dl)	HDL ratio	AI
Control	110.60 ± 1.54	81.49 ± 7.09	11.67 ± 2.42	82.63 ± 1.13	16.30 ± 1.42	12.02 ± 2.84	1.12 ± 0.03
HFD control	143.00 ± 5.16***	108.06 ± 9.86	10.72 ± 0.70	110.67 ± 5.87	21.61 ± 1.97	8.12 ± 0.51	1.08 ± 0.01
Atorvastatin	117.66 ± 5.47	70.86 ± 4.85$$	33.50 ± 7.77	69.99 ± 10.58$	14.17 ± 0.9 7$	45.85 ± 13.63	1.46 ± 0.14
PHF-I 200 mg/kg[a]	112.16 ± 0.76	70.43 ± 0.43$	10.52 ± 0.50	87.55 ± 0.34	14.09±0.09	10.35 ± 0.47	1.10 ± 0.00
PHF-I 400 mg/kg	114.64 ± 6.72	86.80 ± 8.12$$	9.43 ± 0.53	87.85 ± 5.51	17.36 ± 1.62	9.22 ± 0.98	1.09 ± 0.01
PHF-II 200 mg/kg	101.11 ± 0.40	77.95 ± 7.62$$	27.61 ± 7.48	57.91 ± 8.15$$	15.59 ± 1.52	43.07 ± 14.33	1.43 ± 0.14
PHF-II 400 mg/kg	103.58 ± 4.16	76.17 ± 3.54$$$	24.04 ± 8.43	64.30 ± 10.96$$	15.23 ± 0.71	41.29 ± 21.11	1.41 ± 0.21

PHF-I: Pet-ether extract; PHF-II: Methanol extract. All the values are mean ± SEM ($n = 5$)
***$P < 0.001$ as compared to control, One-way repeated measures ANOVA followed by bonferroni post hoc test.
$$P < 0.05$, $$$P < 0.01$, $$$$P < 0.001$ as compared to HFD control, One-way repeated measures ANOVA followed by Bonferroni post hoc test.
[a] $n = 2$.

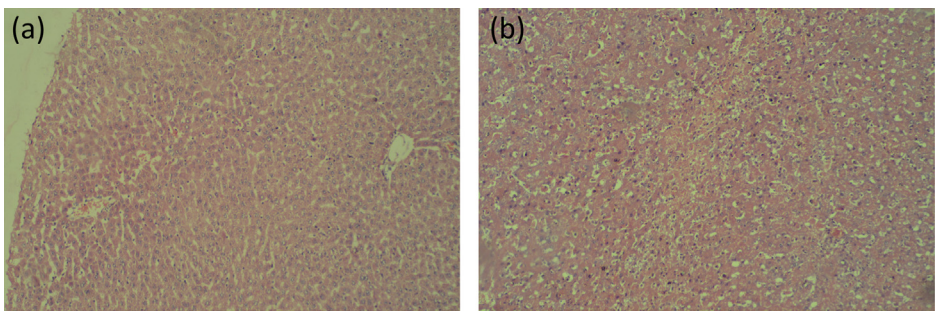

Plate 1. (a) Normal architecture of rat hepatic lobules (×100) H&E stained; (b) Fatty liver tissue with diffuse fatty infiltration, compressed nuclei, and minimal periportal inflammation (×100) H&E stained.

5. Conclusions

The methanol extract of the PHF showed a significant hypolipidemic effect against HFD-induced hyperlipidemia in SD rats. The petroleum ether extract of the PHF did not show a significant hypolipidemic effect against HFD-induced hyperlipidemia in SD rats but produced hyperlocomotion in HDF-fed rats.

Conflicts of interest

All authors have none to declare.

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References

1. Chorghade MS. *Drug Discovery and Development*. New Jersey: John Wiley & Son. Inc; 2007:36.
2. Aronson JK. *Meyler's Side Effect of Herbal Medicines*. San Diego, CA: Elsevier B.V; 2009:3.
3. Spinella M. The importance of pharmacological synergy in psychoactive herbal medicines. *Altern Med Rev*. 2002;7:130–137.
4. Raghavendra HG, Sen S, Reddy YSR, et al. Synergistic activity of *Tribulus terrestris* and *Annona squamosa* extracts against alloxan induced diabetes and hyperlipidemia in rats. *Pharm Sci Mon*. 2011;2:12–23.
5. Parasuraman S, Kumar EP, Kumar A, Emerson SF. Antihyperlipidemia effort if Triglize, a polyherbal formulation. *Int J Pharm Pharm Sci*. 2010;2:118–120.
6. Bhaskar A, Vidhya VG. Hypoglycemic and hypolipidemic activity of *Hibiscus rosa sinensis* Linn on streptozotocin–induced diabetic rats. *Int J Diabetes Dev Ctries*. 2012;32:214–218.
7. Sivaelango G, Senthil KP, Kumaravel P, Revathi P, Jaswant A. Anti-hyperlipidaemic activity of *Spermacoce hispida* ethanolic extract in Triton WR-1339 induced hyperlipidaemic rats. *J Appl Pharm Sci*. 2012;2:95–98.
8. Du H, You JS, Zhao X, Park JY, Kim SH, Chang KJ. Antiobesity and hypolipidemic effects of lotus leaf hot water extract with taurine supplementation in rats fed a high fat diet. *J Biomed Sci*. 2010;17:S42.
9. Mishra S, Dwivedi RR, Ravishankar B, Ashok BK. An experimental Study on hypolipidemic effect of some selected Ruksha Guna drugs. *AYU*. 2009;30:205–210.
10. Subramaniam S, Ramachandran S, Uthrapathi S, Gnamanickam VR, Dubey GP. Anti-hyperlipidemic and antioxidant potential of different fractions of *Terminalia arjuna* Roxb. bark against PX-407 induced hyperlipidemia. *Indian J Exp Biol*. 2011;49:282–288.
11. Petchi RR, Vijaya C, Parasuraman S. Anti-arthritic activity of ethanolic extract of *Tridax procumbens* (Linn.) in Sprague Dawley rats. *Phcog Res*. 2013;5:113–117.
12. Parasuraman S. Toxicological screening. *J Pharmacol Pharmacother*. 2011;2:74–79.
13. Brudzynski SM, Krol S. Analysis of locomotor activity in the rat: parallelism index, a new measure of locomotor exploratory pattern. *Physiol Behav*. 1997;62:635–642.
14. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. *J Pharmacol Pharmacother*. 2010;1:87–93.
15. Mukherjee KL. *Medical Laboratory Technology*. vol. I. New Delhi, India: Tata MC-Graw Hill Publication Company; 1988.
16. Shin MJ, Lee JH, Jang Y, et al. Micellar phytosterols effectively reduce cholesterol absorption at low doses. *Ann Nutr Metab*. 2005;49:346–351.
17. Sudheesh S, Presannakumar G, Vijayakumar S, Vijayalakshmi NR. Hypolipidemic effect of flavonoids from *Solanum melongena*. *Plant Foods Hum Nutri*. 1997;51:321–330.
18. Chen J, Li X. Hypolipidemic effect of flavonoids from Mulberry leaves in Triton WR-1339 induced hyperlipidemic mice. *Asia Pac J Clin Nutr*. 2007;16:290–294.
19. Martín-Castillo A, Castells MT, Adánez G, Polo MT, Pérez BG, Ayala I. Effect of atorvastatin and diet on non-alcoholic fatty liver disease activity score in hyperlipidemic chickens. *Biomed Pharmacother*. 2010;64:275–281.
20. Ghalami J, Zardooz H, Rostamkhani F, Farrokhi B, Hedayati M. High-fat diet did not change metabolic response to acute stress in rats. *EXCLI J*. 2011;10:205–217.
21. Kiley-Worthingto M. *Behavioural Problems of Farm Animals*. Northumberland: Oriel Press Ltd; 1977.
22. Kumar V, Singh P, Chander R, et al. Hypolipidemic activity of *Hibiscus rosa sinesis* root in rats. *Indian J Biochem Biophy*. 2009;46:507–510.
23. Sodipo OA, Abdulrahman F, Sandabe U. Effects of the aqueous fruit extract of *Solanum macrocarpum* Linn. on hematological parameters of chronic Triton-induced hyperlipidemic rats. *J Physiol Pharmacol Adv*. 2012;2:122–132.

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Original article

Phytochemical analysis, HPTLC finger printing, in vitro antioxidant and cytotoxic activity of *Cymodocea serrulata*

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ABSTRACT

Objective: To investigate the phytochemical analysis, HPTLC finger printing, in vitro antioxidant and cytotoxic activity of the methanolic extract of *Cymodocea serrulata* (MECS).
Methods: Phytochemical analysis was carried out to determine the presence of phenol, triterpenoids, flavones, alkaloids, reducing sugars, glycosides, saponins, quinones, proteins, tannins and anthraquinones. HPTLC finger printing was performed using Camag Linomat-V. Antioxidant potential of MECS was determined by DPPH radical scavenging assay, lipid peroxidation inhibition assay and ascorbic acid estimation. Cytotoxic activity was assessed using MTT assay on HeLa cells.
Results: Phytochemical analysis and HPTLC profile of MECS revealed that it possess various phytoconstituents. DPPH radical scavenging assay and lipid peroxidation inhibition assay were tested from 1.5 μg/ml to 1000 μg/ml. The results of these studies showed that at 1000 μg/ml DPPH assay showed 87.81 ± 0.65 inhibition and lipid peroxidation inhibition assay showed 37.15 ± 1.96 inhibition. In cytotoxicity assay, MECS produced maximum percentage of growth inhibition 40.47% at 100 μg/ml.
Conclusion: The results of the present study indicated that MECS possess antioxidant and cytotoxic activity and could be used as a source of antioxidant in the food industry. However further detailed phytochemical, pharmacological and in vivo studies should be the next step in the identification of active constituents.

1. Introduction

Marine organisms offer a vast source to discover useful therapeutics. Recently, numerous marine metabolites with potent pharmacological properties have been identified and many of them already exist in the market.[1] In India seagrass flora is diverse and habitat is limited to mudflats and sandy regions to a depth of ~10–15 m along the open shores and in the lagoons around islands.[2] The roots of *Enhalus acoroides* are used as a remedy against stings of scorpion. *Halophila* species is used to treat malaria, skin diseases and in early stages of leprosy. *Cymodocea* species is used as a tranquilliser for babies, as soothing during pregnancy and in the treatment of cough and malaria.[3] The various parts and extracts of *Cymodocea* species were reported to possess antimicrobial[4–7] and larvicidal property.[8] Many researchers focused their research on marine animals and seaweeds. Generally the seagrasses which are abundantly available are neglected. Hence in this study, the methanolic extract of the seagrass, *Cymodocea serrulata* has been evaluated phytochemical analysis, HPTLC finger printing, in vitro antioxidant activity and cytotoxic activity.

2. Materials and methods

2.1. Collection and authentication of seagrass

The seagrass *C. serrulata* was collected in the coastal area of Rameshwaram during the month of December 2011. The collected seagrass was authenticated by Prof. V. Krishnamurthy, Director, Krishnamurthy Institute of Algology, Anna Nagar, Chennai.

2.2. Extraction of seagrass

C. serrulata initially washed with seawater to remove the macroscopic epiphytes and other extraneous matter and then washed with distilled water. It was shade dried for two weeks and coarsely powdered. The coarsely powdered seagrass was soaked in petroleum ether (1:10) for 72 h with frequent shaking. It was

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Table 1
Phytochemical analysis.

S. no	Chemical test	Result
1	Phenol	+++
2	Triterpenoids	+
3	Flavones	++
4	Alkaloids	+
5	Reducing sugars	+
6	Glycosides	+++
7	Saponins	++
8	Quinones	+
9	Proteins	++
10	Tannins	++
11	Anthraquinones	+

+: Mild, ++: Moderate, +++: High, −: Absent.

filtered using Whatmann filter paper and the petroleum ether extract was obtained. The extract was evaporated to dryness in a water bath. The marc was soaked in n-hexane, chloroform, ethyl acetate and methanol successively for 72 h. Each extract is concentrated by distilling off the solvent and then evaporated to dryness in a water bath. The methanolic extract of *C. serrulata* (MECS) was used to perform preliminary phytochemical analysis, HPTLC finger printing, in vitro antioxidant activity and MTT assay.

2.3. Phytochemical analysis

The methanolic extract of the plant was subjected to phytochemical screening to detect the presence of phenol, triterpenoid, flavone, alkaloid, reducing sugar, glycoside, saponin, quinone, protein, tannin and anthraquinone by the reported protocols.[9]

2.4. HPTLC finger printing of MECS

2.4.1. Instrumentation and chromatographic conditions

Camag Linomat-V (Switzerland) was used as a sample applicator for spotting the samples in the form of bands of 6 mm with Camag microlitre syringe on pre-coated silica gel aluminium plate $60F_{254}$ (10 × 10 cm) with 200 μm thickness (E.Merck, Germany). A constant application rate of 150 nl s^{-1} was employed and space between two bands were 12 mm. The slit dimension was kept at 4.00 × 0.30 mm and 20 mm s^{-1} scanning speed employed.

The composition of mobile phase is toluene:ethyl acetate (8:2). The sample was spotted in a TLC plate and kept on a twin trough glass chamber for linear ascending development. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2 °C). The sample was prepared at the concentration of 10 mg ml^{-1} with methanol. Then 2.5 μl, 5.0 μl, 7.5 μl and 10 μl of sample solution was applied over on pre-coated silica gel $60F_{254}$ TLC plate and developed the plate in the solvent system. Then the developed plate was dried in air and scanned at 254 & 366 nm.

2.5. DPPH radical scavenging assay

DPPH• scavenging potential was measured based on the scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by MECS antioxidants. About 10 μl of MECS solution in various concentrations (1.5–1000 μg/ml) was added to 190 μl DPPH (150 μM) in ethanol solution. After vortexing, the mixture was incubated for 20 min at 37 °C. The decrease in absorbance of the test mixture (due to quenching of DPPH free radicals) was measured at 517 nm.[10]

2.6. Lipid peroxidation inhibition assay

One millilitre of 10% rat liver homogenate was incubated with different concentrations (1.5–1000 μg/ml) of MECS, the lipid peroxidation was initiated by the addition of 0.1 ml of $FeSO_4$ (25 μM), 0.1 ml of ascorbate (100 μM) and 0.1 ml of KH_2PO_4 (10 mM) and the volume was made up to 3 ml with distilled water and incubated at 37 °C for 1 h. Then, 1 ml of 5% trichloroacetic acid and 1 ml of thiobarbituric acid was added to this reaction mixture and

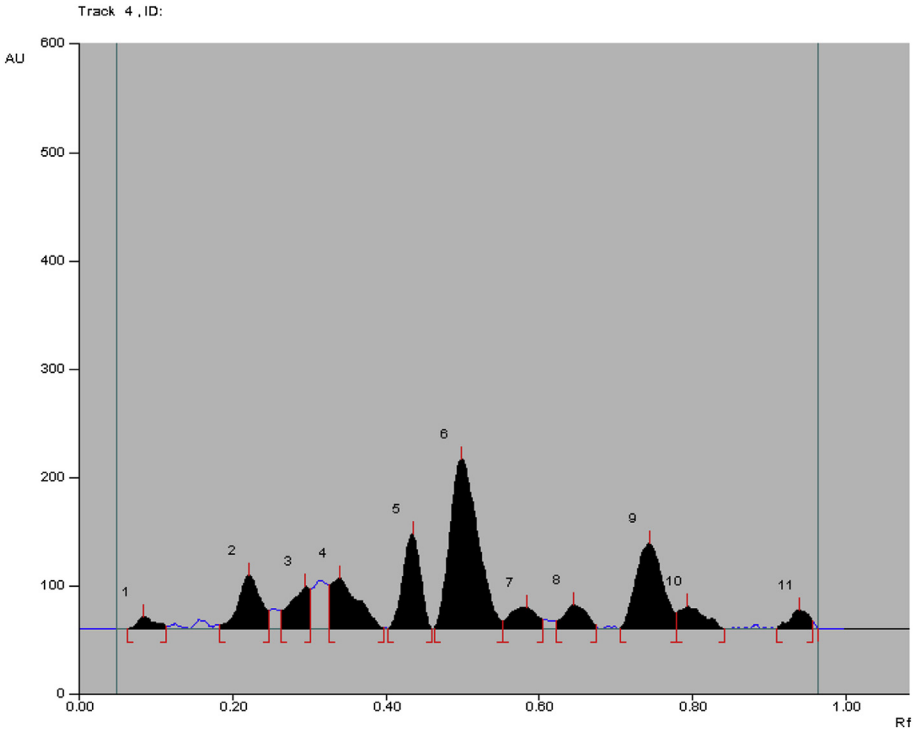

Fig. 1. HPTLC Chromatogram of methanolic extract of *Cymodocea serrulata* (MECS) (10.0 μl).

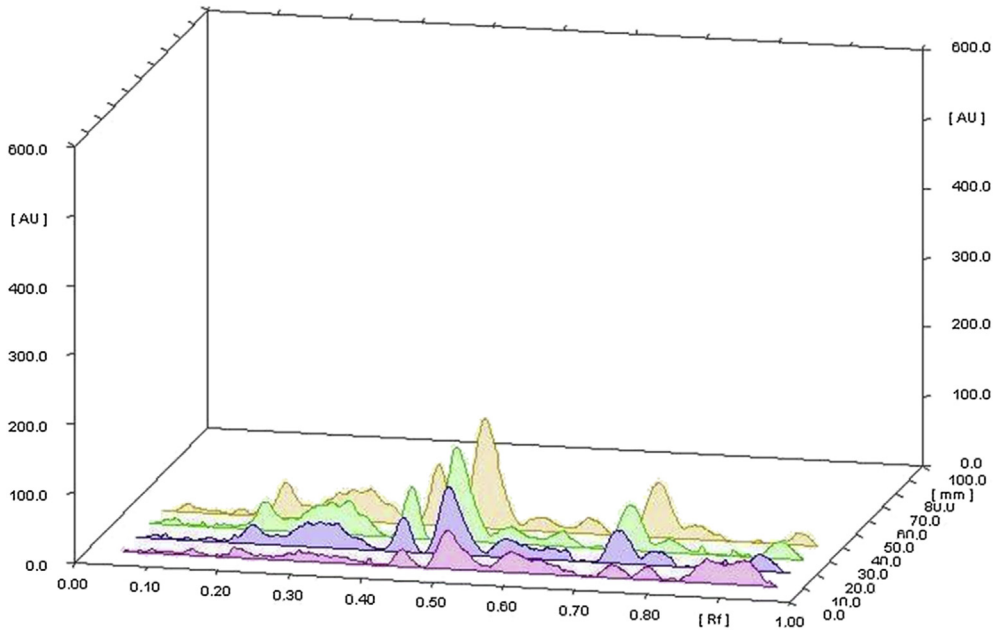

Fig. 2. Three dimensional chromatogram of MECS at 2.5 µl, 5.0 µl, 7.5 µl and 10.0 µl.

the tubes were boiled for 30 min in a boiling water bath. This was centrifuged at 3500 rpm for 10 min. The extent of lipid peroxidation was evaluated by the estimation of thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm.[11]

2.7. Estimation of ascorbic acid

Ascorbic acid was oxidized by copper to form dehydroascorbic acid and diketoglutaric acid. These products were treated with 2,4-dinitrophenylhydrazine to form the derivative of bis-2,4-dinitrophenylhydrazine. This compound in strong sulphuric acid undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm. The reaction was run in the presence of thiourea to provide a mildly reducing medium which helps to prevent interference from non-ascorbic acid chromogens. Aliquots of homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 min at 3500 rpm. 1 ml of the supernatant was mixed with 0.2 ml of DTC reagent (2,4-Dinitrophenylhydrazine-Thiourea-$CuSO_4$ reagent) and incubated for 3 h at 37 °C. Then 1.5 ml of ice-cold 65% H_2SO_4 was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 min. Absorbance was determined at 520 nm. Ascorbic acid values are expressed as % w/w.[12]

2.8. MTT reduction assay on human cervix cancer (HeLa) cells

Cytotoxic activity was assessed using the MTT assay.[13] HeLa cells were seeded in 96-well plates at the density of 10,000 cells/well. Starting concentrations of the extracts were added to the cells and serial dilutions were made. After incubation for 24 h, the MTT solution was added and the plates were incubated again for 2 h. The MTT solution was removed and the formazan product was solubilized using 10% DMSO plus 90% isopropanol. Absorbance was measured at 570 nm using a plate reader (Synergy H_4). The viability was determined based on a comparison with untreated cells. Doxorubicin hydrochloride (Sigma) was used as a positive (cytotoxic) control.

3. Results and discussion

The shade dried, coarsely powdered seagrass, *C. serrulata* was extracted with various solvents such as petroleum ether, n-hexane, chloroform, ethyl acetate and methanol. The yield was higher in methanolic extract of *C. serrulata* than the other extracts. The results of phytochemical study revealed that the MECS has variety of phytochemical constituents such as phenols, triterpenoids, flavones, alkaloids, reducing sugars, glycosides, saponins, quinones, proteins, tannins and anthraquinones (Table 1). The present study also included the HPTLC finger printing of MECS. The applied

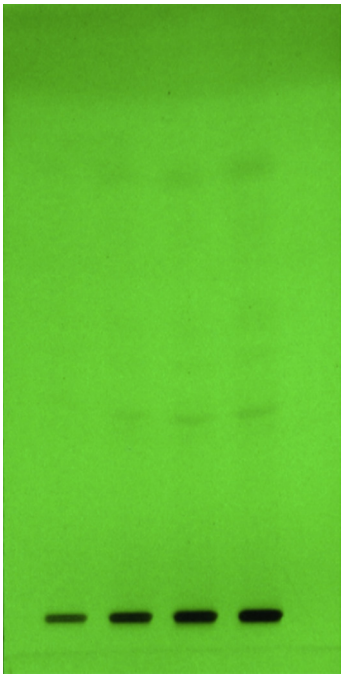

Fig. 3. HPTLC chromatographic plate of MECS viewed under 254 nm.

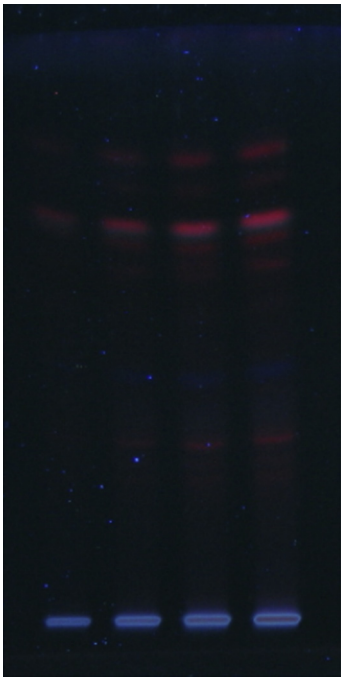

Fig. 4. HPTLC chromatographic plate of MECS viewed under 366 nm.

volumes were 2.5 µl, 5.0 µl, 7.5 µl and 10.0 µl. The three dimensional and individual chromatograms at various applied volumes were also recorded (Figs. 1 and 2). The developed TLC plate was scanned at 254 nm and 366 nm. At 366 nm many bright coloured spots were also observed (Figs. 3 and 4).

The antioxidant potential of MECS was evaluated using different bioassays such as DPPH scavenging assay, lipid peroxidation inhibition assay and ascorbic acid estimation. DPPH radical scavenging assay activity increased with increasing amount of concentration. But in lipid peroxidation inhibition assay the activity increases with increasing amount of concentration up to 500 µg/ml. Beyond that concentration there was no increase in the activity (Table 2). 0.024% w/w concentration of ascorbic acid content was also reported and the values were expressed as Mean ± SEM ($n = 3$).

Cytotoxicity was assessed using the MTT assay on human cervix cancer (HeLa) cells. Doxorubicin hydrochloride was used as a positive (cytotoxic) control. The standard produced dose dependent cytotoxicity on HeLa cells and it produces the maximum percentage of growth inhibition (98.27%) at 100 µg/ml. Whereas the test compound, MECS produced the maximum percentage of growth inhibition (40.57%) at 100 µg/ml at all the dose levels tested (0.01 µg/ml–100 µg/ml) (Table 3).

Table 2
In vitro antioxidant assays.

Concentration (µg/ml)	DPPH	LPO
1.5	1.03 ± 0.84	–
3	3.28 ± 0.87	–
7	5.73 ± 0.56	1.84 ± 1.73
15	8.77 ± 0.21	2.09 ± 1.65
30	21.82 ± 2.13	11.18 ± 1.11
60	27.84 ± 1.33	14.72 ± 1.31
125	42.00 ± 3.76	13.18 ± 1.45
250	46.24 ± 3.19	24.44 ± 1.26
500	72.35 ± 5.77	37.54 ± 2.12
1000	87.81 ± 0.65	37.15 ± 1.96

Table 3
Growth inhibition by test compound (MECS) and standard compound (Doxorubicin) on HeLa cell line.

Concentration (µg/ml)	% Growth inhibition	
	Test	Standard
100	40.57	98.27
10	25.38	97.67
1	13.57	48.74
0.1	5.00	38.79
0.01	0.57	17.70

4. Conclusion

The present study suggested that the MECS possess antioxidant and cytotoxic activity. The results showed that phenols and flavones could have played a major role in the DPPH assay since the amount of the compounds were proportionate to the DPPH scavenging activity. Hence MECS might be helpful in preventing or slowing the progress of various oxidative stress-related diseases. Further investigation on the isolation and identification of antioxidant and cytotoxic component of the plant may lead to chemical entities with potential for clinical use.

Conflicts of interest

All authors have none to declare.

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References

1. Thakur Narsinh L, Thakur Archana N, Muller Werner EG. Marine natural products in drug discovery. *Nat Prod Radiance*. 2005;4:471–477.
2. Jagtap Tanajii G, Komarpant Deepali S, Rodrigues Rouchelle S. Status of a seagrass ecosystem: an ecologically sensitive wetland habitat from India. *Wetlands*. 2003:161–170.
3. Kumar Chinnadurai Sreenath, Sarada Dronamraju VL, Gideon Thomas Paul, Rengasamy Ramasamy. Antibacterial activity of three south Indian seagrasses, *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capensis*. *World J Microbiol Biotechnol*. 2008;24:1989–1992.
4. Mayavu P, Sugesh S, Ravindran VJ. Antibacterial activity of seagrass species against biofilm forming bacteria. *Res J Microbiol*. 2009;4:314–319.
5. Kannan Rengasamy Ragupathi Raja, Arumugam Rajasekaran, Anantharaman Perumal. Antibacterial potential of three seagrasses against human pathogens. *Asian Pac J Trop Med*. 2010:890–893.
6. Ravikumar Sundaram, Nanthini devi K, Ajith kumar TT, Ajmalkhan M. Antibacterial activity of seagrass species of *Cymodocea serrulata* against chosen bacterial fish pathogens. *Ann Biol Res*. 2011;2:88–93.
7. Ravikumar S, Syed Ali M, Anandh P, Ajmalkhan M, Dhinakaraj M. Antibacterial activity of *Cymodocea serrulata* root extract against poultry pathogens. *Indian J Sci Technol*. 2011;4:98–100.
8. Syed Ali M, Ravikumar S, Margaret Beula J. Bioactivity of seagrass against the dengue fever mosquito *Aedes aegypti* larvae. *Asian Pac J Trop Biomed*. 2012:1–5.
9. Kokate CK. *Practical Pharmacognosy*. 4th ed. Delhi: Vallabh Prakashan; 2006.
10. Koleva I, Van Beek TA, Linssen JPH, De Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochem Anal*. 2002;13:8–17.
11. Ohkawa H, Ohishi N, Yogi K. Assay of lipid peroxides in animal tissue by thiobarbituric reactions. *Anal Biochem*. 1979;95:351–358.
12. Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol*. 1979;62:3–11.
13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 1983;65:55–63.

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Review article

Botany, uses, phytochemistry and pharmacology of *Vallaris*: A short review

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ABSTRACT

The World Checklist of Selected Plant Families has listed a global list of 28 species for *Vallaris* under the family Apocynaceae of which only *Vallaris glabra*, *Vallaris solanacea* and *Vallaris indecora* are accepted names. In this short review, the current knowledge of the botany, uses, phytochemistry and pharmacology of *V. glabra* and *V. solanacea* is reviewed as there is no information on the chemical constituents and bioactivities of *V. indecora*. *V. glabra* (L.) Kuntze or bread flower is a woody climber with broadly elliptic leaves. Flowers are cup-like and white with a unique fragrance of leaves of pandan or newly cooked fragrant rice. The species is a popular ornamental plant in gardens of Southeast Asia. No uses of *V. glabra* in traditional medicine have been reported. From the leaves of *V. glabra*, cardiac glycosides, phenolic acids, fatty acids and triterpenes have been isolated. Essential oils extracted from flowers have been identified. The antiproliferative, antiplasmodial and antioxidant properties of *V. glabra* are reviewed. *V. solanacea* (Roth) Kuntze is a twining shrub up to 10 m tall. Leaves are elliptic and densely pubescent on both surfaces. Flowers are white or creamy, fragrant and borne in clusters. The species occurs naturally in forests of South and Southeast Asia. Traditionally, the milky latex of *V. solanacea* can be applied to treat ringworm and other skin infections, including sores, cuts and wounds. From leaves and seeds of *V. solanacea*, cardiac glycosides, fatty acids and triterpenes have been isolated. From the root bark, essential oils have been identified. Leaves and barks of *V. solanacea* have been reported to possess anticancer, antimicrobial, analgesic, anti-inflammatory, anti-diarrhoeal and cardiotonic properties, and display toxicity to brine shrimp but not to rats. *V. indecora* (Baill.) Tsiang & P.T. Li is a trailing shrub with elliptic or obovate leaves and pale yellow flowers. Occurring in China, the plant is used to treat worm diseases.

1. Introduction

In the World Checklist of Selected Plant Families developed by the Royal Botanic Gardens at Kew, England, a total of 28 botanical names of *Vallaris* species have been listed under the family Apocynaceae of which only *Vallaris glabra*, *Vallaris solanacea* and *Vallaris indecora* are accepted names.[1] In this short review, the current knowledge of the botany, uses, phytochemistry and pharmacology of *V. glabra* and *V. solanacea* is reviewed. No information is available in the literature on the chemical constituents and bioactivities of *V. indecora*.

V. glabra (L.) Kuntze or bread flower is a woody climber with broadly elliptic leaves of 7–9 × 4–6 cm in size.[2] Inflorescences are long-stalked with clusters of fragrant cup-like white flowers of 1.0–1.5 cm in diameter (Fig. 1). The plant is well known in Thailand and Malaysia because its flowers have a unique scent of leaves of pandan (*Pandanus amaryllifolius*) or newly cooked fragrant rice. Originated from Java in Indonesia, the species grows in full sun and can be propagated by marcotting. With its attractive clusters of white cup-like flowers that emit a strong pandan fragrance, *V. glabra* has become a popular ornamental plant in botanic and home gardens of Southeast Asia. There are no reports on the use of this species in traditional medicine.

V. solanacea (Roth) Kuntze is a climbing shrub, often twining and can grow up to 10 m tall.[3] Leaves are elliptic and densely pubescent on both surfaces. Flowers are white or creamy, fragrant and borne in clusters (Fig. 2). The species occurs naturally along stream banks in forests of South and Southeast Asia. Traditionally, the milky latex of *V. solanacea* can be applied to treat ringworm and other skin infections, including sores, cuts and wounds.[4,5]

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Fig. 1. Flowers and leaves of *Vallaris glabra*.

V. indecora (Baill.) Tsiang & P.T. Li is a trailing shrub with pale grey bark that can grow up to 6 m tall.[3] Leaves are elliptic or obovate with 6–8 pairs of lateral veins. Each inflorescence has 3 or 4 pale yellow flowers. The species occurs at altitudes of 700–3000 m asl in Guangxi, Guizhou, Sichuan and Yunnan of China. All parts are used to treat for worm diseases.

2. Phytochemistry

2.1. Cardiac glycosides

Bioassay-guided separation of *V. glabra* leaves has led to the isolation of two cardiac glycosides.[6] One was identified as acoschimperoside P, 2′-acetate, a known compound first reported in seeds of *V. solanacea*[7] and the other was new.

From seeds of *V. solanacea*, glycosides of vallaroside, solanoside, vallarosolanoside, 16-deacetyl-16-anhydro-acoschimperoside P, mono-*O*-acetyl-acoschimperoside P, mono-*O*-acetyl-vallaroside and mono-*O*-acetyl-solanoside have been reported.[7] *O*-Acetyl-solanoside was isolated from leaves of *V. solanacea*.[8] Subsequent chemical investigations reported β-sitosterol, β-amyrin, ursolic acid, vallaroside, solanoside, vallarosolanoside and acoschimperoside P from leaves.[4] A new cardenolide glycoside of vallarisoside and a known cardenolide glycoside of 3β-*O*-(α-acofriosyl)-16-anhydrogitoxigenin, along with a new glycoside of benzyl 2-*O*-β-apiofuranosyl-(1 → 2)-β-D-glucopyranosyl-2,6-dihydroxy-benzoate have also been isolated.[4,9] Their molecular formulae were $C_{32}H_{46}O_9$, $C_{30}H_{44}O_8$ and $C_{25}H_{30}O_{13}$ with molecular weights of 597.3, 571.0 and 561.2, respectively. The known cardenolide glycoside was previously reported as 16-desacetyl-16-anhydro-acoschimperosid P.[7]

Fig. 2. Flowers and leaves of *Vallaris solanacea* (photo by J.M. Garg).

2.2. Caffeoylquinic acids

From the MeOH leaf extract of *V. glabra*, 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid, 4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid, and 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid (CGA) were isolated.[10,11]

Caffeoylquinic acids are esters of caffeic and quinic acids. 3-CQA, 4-CQA and 5-CQA or CGA have the caffeoyl group attached to carbons 3, 4 and 5 of the quinic moiety, respectively. They have a similar molecular formula of $C_{16}H_{18}O_9$ and molecular weight of 354. The isolation of CQAs from leaves of *V. glabra* was the first for the genus *Vallaris*.[10,11] Earlier studies have documented the occurrence of CQAs in other Apocynaceae species. 3-CQA and 5-CQA have been isolated from stems and leaves of *Catharanthus roseus*, and 4-CQA from petals.[12]

The content of 5-CQA or CGA in leaves of *V. glabra* (353 ± 25 mg CGA/100 g) was two times higher than flowers of *Lonicera japonica* or Japanese honeysuckle (173 ± 13 mg CGA/100 g), the commercial source of CGA.[10,11] The 3-CQA and 4-CQA content in leaves of *V. glabra* (370 ± 15 mg CGA/100 g) was 16 times higher than flowers of *L. japonica* (23 ± 2.2 mg CGA/100 g). Compared with other plants, the CGA content in leaves of *V. glabra* is significantly higher than leaves of *Etlingera elatior* (torch ginger) and *Ipomoea batatas* (sweet potato) with values of 294 ± 25 and 115 ± 16 mg CGA/100 g, respectively.[13,14]

2.3. Other compounds

From the DCM leaf extract of *V. glabra*, stearic acid (SA) and ursolic acid (UA) have been isolated.[15] SA or octadecanoic acid, with molecular formula of $C_{18}H_{36}O_2$ and molecular weight of 284.5 is a saturated fatty acid with an 18-carbon chain. Ursolic acid is a pentacyclic triterpene acid with molecular formula of $C_{30}H_{48}O_3$ and molecular weight of 456.7.

SA isolated from leaves of *V. glabra* has been reported in other Apocynaceae species such as leaves and flowers of *C. roseus*,[16] and leaves of *Alstonia boonei*.[17] In Apocynaceae, UA has been isolated from *Alstonia scholaris*[18] and *Plumeria rubra*.[19] Widely found in plants, UA is a compound that possesses many biological activities such as antioxidative, anti-inflammatory, anticancer and hepatoprotective, as well as the ability to induce apoptosis.[20]

A phytochemical study on flowers of *V. glabra* revealed the presence of monoterpenes, sesquiterpenes and acyclic monoterpene alcohols.[2] Linalool (62%) was found to be the principal constituent. Of particular interest is the occurrence of 2-acetyl-1-pyrroline (2AP). The aromatic compound was first reported in cooked rice and in leaves of pandan. Using capillary GC, the content of 2AP in flowers (3.4 mg/kg) was comparable to Thai fragrant rice (3.0 mg/kg) but lower than pandan leaves (10 mg/kg).

The composition and content of fatty acids from the root bark of *V. solanacea* have been studied.[21] Eight types of fatty acids were identified with arachidonic acid (42%), palmitic acid (38%) and capric acid (15%) as the main components.

3. Pharmacology

3.1. Anticancer properties

Isolated from leaves of *V. glabra*, acoschimperoside P, 2′-acetate was active in hedgehog (Hh) signalling inhibition.[6] The cardiac

glycoside inhibited Hh/GLI-mediated transcriptional activity with an IC_{50} value of 2.3 μM. The compound also showed strong cytotoxicity against human pancreatic PANC1 (IC_{50} of 3.6 μM) and human prostate DU145 cancer cells (IC_{50} of 1.8 μM). The cytotoxic effect was associated with the ability of the compound to inhibit the Hh/GLI1 signalling pathway. The compound was found to inhibit the levels of GLI-related proteins of patched (PTCH) and B-cell lymphoma 2 (BCL-2) in PANC1 cells.

The antiproliferative activity of sequential extracts of different plant parts of *V. glabra* has been reported.[22,23] DCM and DCM:MeOH leaf extracts, and DCM flower extract displayed broad-spectrum antiproliferative activity with effective inhibition of HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells (Table 1). Inhibition of stem extracts was more specific to MCF-7 and SKOV-3 cells with no activity against MDA-MB-231 cells. Against MCF-7 cells, the GI_{50} values of DCM extracts of stems and flowers (both 1.4 ± 0.2 μg/ml) were lower than that of the positive controls. DCM and DCM:MeOH extracts of leaves were comparable to tamoxifen. Hexane extracts of stems and flowers were more potent than tamoxifen and xanthorrhizol. Overall, the screening results showed that extracts from three different parts of *V. glabra* possessed effective antiproliferative activity. Other Apocynaceae species that displayed positive antiproliferative activity were *Alstonia angustiloba*, *Calotropis gigantea*, *C. roseus*, *Nerium oleander* and *Plumeria obtusa*.

MDA-MB-231 cancer cells treated with DCM leaf extract of *V. glabra* and stained with Hoechst 33342 dye showed that the extract had an apoptotic effect on the cells.[11,15] Cells treated with DCM leaf extract at 12.5, 25.0 and 50.0 μg/ml displayed apoptotic morphology (Fig. 3).

MDA-MB-231 cancer cells were treated with concentrations varying from 1.6 to 25 μg/ml of DCM leaf extract of *V. glabra*.[11,15] A histogram of fold-increase in caspase activity vs. concentration of DCM leaf extract is shown in Fig. 4. Based on caspase colorimetry, the apoptotic effect involved activation of caspase-8, -9 and -3, but not caspase-6. This indicates that the extract induced apoptosis through both the extrinsic (death-receptor) and intrinsic (mitochondrial) pathways. Caspase-8 and -9 are initiator caspases, while caspase-3 and -6 are effector caspases.

Of the isolates from leaves of *V. solanacea*, vallarisoside showed potent TRAIL-resistance-overcoming activity in human gastric adenocarcinoma (AGS) cells and cell growth-inhibitory activity against HeLa and SW480 cells.[9]

Table 1
Antiproliferative activity of sequential extracts of *Vallaris glabra* against four human cancer cell lines.[22,23]

Plant part	Sequential extract	Growth inhibition (GI50)			
		MCF-7	MDA-MB-231	HT-29	SKOV-3
Leaf	Hexane	–	–	–	–
	DCM	7.7 ± 1.3	12 ± 2.0	7.5 ± 4.5	9.3 ± 2.0
	DCM:MeOH	7.0 ± 2.5	13 ± 6.3	7.7 ± 2.4	12 ± 1.2
	MeOH	16 ± 2.1	–	–	–
Stem	Hexane	7.0 ± 0.6	–	–	1.8 ± 0.3
	DCM	1.4 ± 0.2	–	1.7 ± 0.5	1.2 ± 0.1
	DCM:MeOH	3.9 ± 0.7	–	–	1.7 ± 0.3
	MeOH	5.4 ± 1.6	–	–	1.8 ± 0.2
Flower	Hexane	3.3 ± 1.0	–	5.9 ± 2.0	–
	DCM	1.4 ± 0.2	4.4 ± 1.2	1.4 ± 0.3	1.6 ± 0.2
	DCM:MeOH	4.6 ± 1.1	–	6.5 ± 1.1	3.1 ± 0.7
	MeOH	–	–	–	7.4 ± 0.8
Xanthorrhizol		11 ± 0.7	8.7 ± 0.8		
Curcumin		4.1 ± 0.9	8.7 ± 0.8		
Tamoxifen		8.3 ± 0.6	4.6 ± 0.5		

GI_{50} (μg/ml) is the concentration which causes 50% reduction in cell growth and inhibition is not effective (–) if values ≥20 μg/ml. MCF-7 and MDA-MB-231 are human breast cancer cells, HeLa and SKOV-3 are human cervical cancer cells, HT-29 are human colon cancer cells and HepG2 are human liver cancer cells. Values of standard drugs of xanthorrhizol, curcumin and tamoxifen against MCF-7 and MDA-MB-231 were used as positive controls. Abbreviations: MeOH = methanol and DCM = dichloromethane.

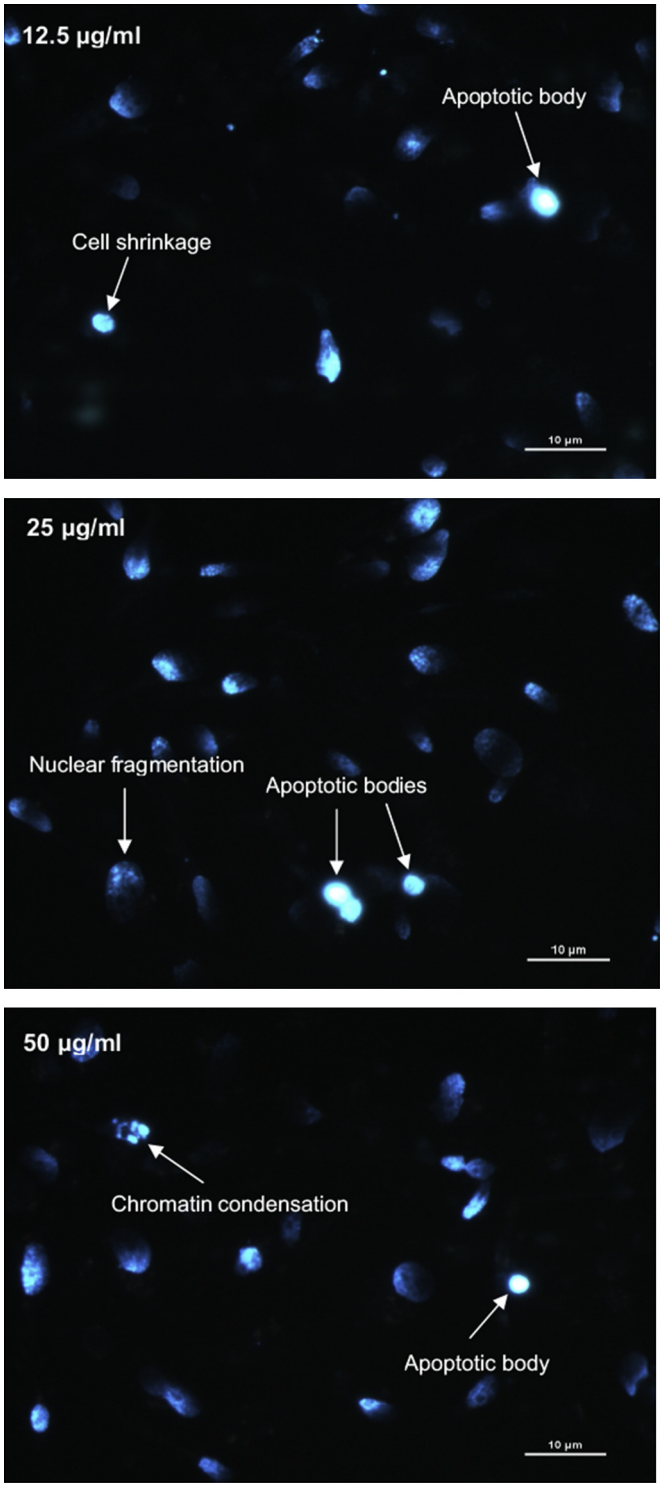

Fig. 3. DCM leaf extract of *Vallaris glabra* at 12.5, 25.0 and 50.0 μg/ml showed apoptotic effects on treated MDA-MB-231 breast cancer cells.[11,15]

3.2. Antimalarial properties

The antiplasmodial activity of leaf extracts of *V. glabra* has been reported.[11,22] All hexane, DCM, DCM:MeOH and MeOH leaf extracts of *V. glabra* displayed effective antiplasmodial activity against

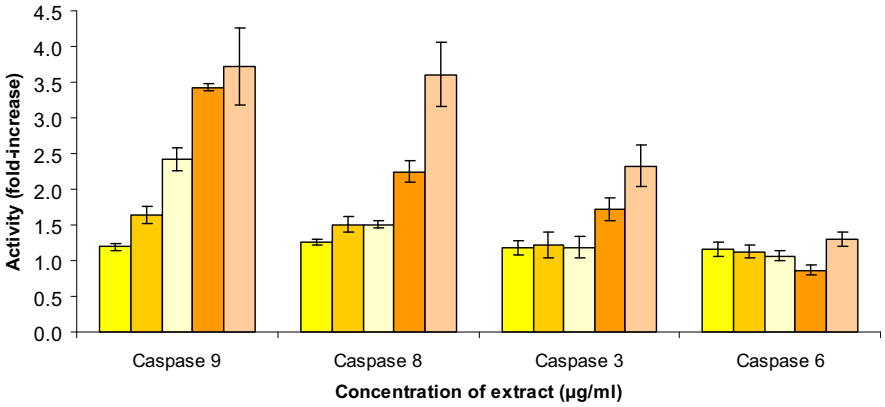

Fig. 4. Histogram of fold-increase in caspase-9, -8, -3 and -6 activity vs. concentrations of DCM leaf extract of *Vallaris glabra*.[11,15] For each caspase, bars from left to right are extract concentrations at 1.56, 3.13, 6.25, 12.5 and 25.0 µg/ml.

chloroquine-resistant K1 strain of *Plasmodium falciparum* with EC_{50} values of 1.0, 0.8, 8.5 and 8.4 µg/ml, respectively. No activity was observed against chloroquine-sensitive 3D7 strain of *P. falciparum*. The selectivity index (SI) for antiplasmodial activity against K1 strain suggested that the extracts of *V. glabra* are potentially safe for use to treat malaria.

In general, EC_{50} values of leaf extracts against K1 strain of *P. falciparum* were much weaker than standard drugs of artemisinin and mefloquine.[22] Other Apocynaceae species reported to have antiplasmodial activity were *A. angustiloba*, *C. gigantea*, *Dyera costulata* and *Kopsia fruticosa*.

3.3. Antioxidant properties

Out of methanol leaf extracts of six Apocynaceae species screened for antioxidant properties, *V. glabra* ranked second to *D. costulata*.[22] Values of total phenolic content, free radical scavenging activity and caffeoylquinic acid content of *V. glabra* were 1300 ± 120 mg GAE/100 g, 1000 ± 100 mg AA/100 g and 380 ± 50 mg CGAE/100 g of samples (fresh weight).

3.4. Antimicrobial properties

At 40 µg/ml, the stem bark extract of *V. solanacea* has been demonstrated to inhibit the growth of Gram-positive bacteria of *Staphylococcus aureus* and *Bacillus subtilis*, Gram-negative bacteria of *Salmonella typhi* and *Escherichia coli*, and fungi of *Candida albicans* and *Aspergillus niger*.[24] Petroleum ether, chloroform and ethanol extracts were tested using the agar well diffusion method. All solvent extracts yielded positive results with the petroleum ether extract exhibiting relatively higher zone of inhibition against *S. typhi*, *E. coli*, *A. niger* and *C. albicans*. Inhibition was comparable to that of standard drugs of ciprofloxacin and fluconazole (40 µg in 100 µl).

3.5. Analgesic properties

Ethanol extracts of leaves and stems of *V. solanacea* showed significant analgesic activity in acetic acid-induced writhing inhibition in mice.[5] Writhing inhibition at a dose of 500 mg/kg body weight was 54%. At 250 mg/kg body weight, writhing inhibition was 23%. Ethanol bark extract of *V. solanacea* was also tested for analgesic activity.[25] Writhing inhibition was 52% and 23% at doses of 500 and 250 mg/kg body weight, respectively.

3.6. Anti-inflammatory activity

Using the carrageenin-induced rat-paw oedema model, ethanol bark extract of *V. solanacea* significantly reduced the paw volume from 1 to 5 h.[25] The extract showed highest effects at the third hour when the inhibition was 29% and 41% at doses of 200 and 400 mg/kg respectively.

3.7. Anti-diarrhoeal activity

The anti-diarrhoeal activity of ethanol bark extract of *V. solanacea* was demonstrated by castor oil-induced diarrhoea in mice.[25] Results showed that 500 mg/kg body weight of extract significantly delayed the onset of diarrhoea episode to 1.1 h compared to 0.7 h of the control. The number of stools excreted by the animals (per group of 5) after 4 h was 1.3 and 3.6, respectively.

3.8. Cardiotonic activity

O-Acetyl-solanoside, a cardiac glycoside, isolated from leaves of *V. solanacea* was found to possess marked cardiotonic activity in cats and guinea pigs.[8] Its therapeutic index is comparable to that of ouabain. The compound has a quick onset, medium duration of action, and shows consistent and dependable oral absorption with low cumulative toxicity.

3.9. Toxicity

A toxicity study of essential oil from the root bark of *V. solanacea* has been carried out on albino rats.[21] Two groups of rats weighing 150–200 g were orally administered with the oil at doses of 3 ml/kg and 5 ml/kg body weight. None of the rats showed any toxicity effects of mortality after 12 h. The ethanol extract of leaves and stems of *V. solanacea* showed brine shrimp toxicity at LC_{50} of 80 µg/ml and LC_{90} of 320 µg/ml.[5]

4. Conclusion

Of the two *Vallaris* species reviewed, the wide array of pharmacological properties of leaves and barks of *V. solanacea* confers its use in traditional medicine. Properties include antiproliferative, antimicrobial, analgesic, anti-inflammatory, anti-diarrhoeal and cardiotonic activities. The potent TRAIL-resistance-overcoming activity in AGS cancer cells and cell growth-inhibitory activity against HeLa and SW480 cells of vallarisoside isolated from leaves of

V. solanacea deserve further research. *V. glabra* is a unique plant because it displayed both strong and broad-spectrum anti-proliferative activity against HT-29, MCF-7, MDA-MB-231 and SKOV-3 cancer cells, and antiplasmodial activity against K1 strain of *P. falciparum*. Acoschimperoside P, 2′-acetate, a cardiac glycoside isolated from leaves of *V. glabra* inhibited Hh/GLI1 signalling pathway and showed strong cytotoxicity against PANC1 and DU145 cancer cells is noteworthy. The species would be of interest to the pharmaceutical industry as a potential candidate for anticancer and antimalarial drug discovery. With much higher CQA content in the leaves of *V. glabra* than flowers of *L. japonica* (the commercial source), the species can serve as a promising alternative source of CQA.

Conflicts of interest

All authors have none to declare.

References

1. WCSP. *Vallaris*. Royal Botanic Gardens, Kew: World Checklist of Selected Plant Families; 2010. Cited 12 September 2013; Available from: http://www.theplantlist.org/browse/A/Apocynaceae/Vallaris.
2. Wongpornchai S, Sriseadka T, Choonvisase S. Identification and quantitation of the rice aroma compound, 2-acetyl-1-pyrroline, in bread flowers (*Vallaris glabra* Ktze). *J Agric Food Chem*. 2003;51:457–462.
3. Li B, Leeuwenberg AJM, Middelton DJ. Vallaris. *Flora China*. 1995;16:177.
4. Ahmed F, Sadhu SK, Ishibashi M. Search for bioactive natural products from medicinal plants of Bangladesh. *J Nat Med*. 2010;64:393–401.
5. Karmakar KU, Ghosh D, Sadhu SK. Assessment of analgesic, cytotoxic and antioxidant activities *Vallaris solanacea* (Roth) Kuntze. *Stamford J Pharm Sci*. 2011;4:64–68.
6. Rifai Y, Arai MA, Koyano T, Kowithayakorn T, Ishibashi M. Aco-schimperoside P, 2′-acetate: a hedgehog signalling inhibitory constituent from *Vallaris glabra*. *J Nat Med*. 2011;65:629–632.
7. Kaufmann H. The glycosides of the seeds of *Vallaris solanacea* (Roth) clarification of structure, glycosides and aglycones. *Helv Chim Acta*. 1965;48:83–94.
8. Vohra MM, Patnaik GK, Kapil RS, Anand N. Chemistry and pharmacology of a glycoside of *Vallaris solanacea*. *J Pharm Sci*. 1966;55:1425–1428.
9. Ahmed F, Sadhu SK, Ohtsuki T, Khatun A, Ishibashi M. Glycosides from *Vallaris solanaceae* with TRAIL-resistance-overcoming activity. *Heterocycles*. 2010;80:477–488.
10. Wong SK, Lim YY, Ling SK, Chan EWC. Caffeoylquinic acids in leaves of selected Apocynaceae species: their isolation and content. *Pharmacogn Res*. 2014 (In Press).
11. Wong SK, Lim YY, Chan EWC. Botany, uses, phytochemistry and pharmacology of selected Apocynaceae species: a review. *Pharmacogn Commn*. 2013;3:2–11.
12. Ferreres F, Pereira DM, Valentao P, Andrade PB, Seabra RM, Sottomayor M. New phenolic compounds and antioxidant potential of *Catharanthus roseus*. *J Agric Food Chem*. 2008;56:9967–9974.
13. Chan EWC, Lim YY, Ling SK, Tan SP, Lim KK, Khoo MGH. Caffeoylquinic acids from leaves of *Etlingera* species (Zingiberaceae). *LWT – Food Sci Technol*. 2009;42:1026–1030.
14. Chan EWC, Lim YY, Wong SK. Phytochemistry and pharmacological activities of *Etlingera elatior*: a review. *Pharmacogn J*. 2011;3:6–10.
15. Wong SK, Lim YY, Ling SK, Chan EWC. Antiproliferative activity of *Vallaris glabra* (L.) Kuntze (Apocynaceae). *Pharmacogn Mag*. 2014 (In Press).
16. Pandey-Rai S, Mallavarapu GR, Naqvi AA, et al. Volatile components of leaves and flowers of periwinkle *Catharanthus roseus* (L.) G. Don from New Delhi. *Flavour Frag J*. 2006;21:427–430.
17. Okwu DE, Ighodaro BU. GC-MS evaluation of bioactive compounds and antibacterial activity of the oil fraction from the leaves of *Alstonia boonei* De Wild. *Der Pharm Chem*. 2010;2:261–272.
18. Shetty P, Mangaonkar K, Sane RT. HPTLC determination of ursolic acid in *Alstonia scholaris* R Br. *J Planar Chromatogr – Mod TLC*. 2007;20:65–68.
19. Silva JA, Silva AG, Alves AS, et al. *Plumeria rubra* (Apocynaceae): a good source of ursolic acid. *J Med Plant Res*. 2013;7:892–896.
20. Kim KA, Lee JS, Park HJ, et al. Inhibition of cytochro P450 activities by oleanolic acid and ursolic acid in human liver microsomes. *Life Sci*. 2004;74:2769–2779.
21. Punam J, Bhanu P, Manoj G. Fatty acid composition and physico-chemical properties of the oil extracted from root bark of *Vallaris solanacea* (Roth) Kuntze. *Int Res J Pharm*. 2012;3:165–166.
22. Wong SK, Lim YY, Abdullah NR, Nordin FJ. Antioxidant, antiproliferative and antiplasmodial activities of leaves of selected Apocynaceae species. *BMC Complement Altern Med*. 2011;11:3.
23. Wong SK, Lim YY, Abdullah NR, Nordin FJ. Antiproliferative and phytochemical analyses of leaf extracts of ten Apocynaceae species. *Pharmacogn Res*. 2011;3:100–106.
24. Vagdevi HM, Hanumanthappa BC, Vaidya VP, Raghavendra R, Parashurama TR. *In-vitro* antibacterial and antifungal evaluation of *Vallaris solanacea* (Roth) Kuntze. *Int J Pharm Res Dev*. 2011;3:29–33.
25. Ashikur R, Ekramul H, Hasanuzzaman MD, Muhuri SR, Shahid IZ. A study on antinociceptive, antiinflammatory and antidiarrhoeal activities of *Vallaris solanacea* (Roth) Kuntze bark extracts. *J Pharm Allied Sci*. 2011;8:1268–1275.