Protective Effects of Beetroot Extract against Phenyl Hydrazine Induced Anemia in Rats

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ABSTRACT

Background: Anemia can be regarded as one of the oldest blood malformation known over a century. Megaloblastic anemia arises due to curtailed formation of erythrocytes, which leads to formation of a large number of underdeveloped erythrocytes. Proper nutritional supplementation may be useful in such condition. *Beta vulgaris* or beetroot is one of the important vegetables consumed worldwide. The aim of the present work was to evaluate anti-anemic potential of beetroot. **Methods:** Beetroot was extracted with ethanol. Phytochemical and phytoanalytical studies were performed on extract. Anemia was induced by phenyl-hydrazine. Animals were treated with extract throughout the study for 24 days. The red blood cell (RBC) number and hemoglobin concentration were determined every 3 days for 24 days. **Results:** Extract was found to be rich in folic acid, ascorbic acid, and iron. Following the induction of anemia, the number of erythrocytes and the hemoglobin concentration decreased by 62.51% and 69.64%, respectively. Administration of standard hematinic preparation and extract (200 mg/kg) resulted in significant increase ($P < 0.001$; $P < 0.01$) in the number of RBCs as well as hemoglobin concentration when compared to the untreated phenyl hydrazine-induced anemic rats. **Conclusion:** Extract effectively raised the level of hemoglobin and erythrocyte count at dose 200 mg/kg. Vitamin and minerals found in beetroot are most likely active ingredients responsible for its hematinic effects. Still, methodical studies are obligatory to derive its effects on humans.

Keywords: *Beta vulgaris*, erythrocytes, hemoglobin, phenyl hydrazine

INTRODUCTION

Anemia can be regarded as one of the oldest blood malformation known over a century.[1] It is a disorder of blood cells characterized by manifestation of giant erythrocytes. Megaloblastic anemia arises due to curtailed formation of erythrocytes, which leads to formation of a large number of underdeveloped erythrocytes. Such erythrocytes cannot function like normal one; such cells also have short lifespan. Folic acid deficiency could be considered as a major cause of this malformation. Proper nutritional supplementation may be useful in such condition.

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Beta vulgaris or beetroot is one of the important vegetable consumed worldwide. Scientifically, beetroot is recognized to increase exercise stamina[2] and increases running performance.[3] it is also utilized in management of hypertension.[4,5] It is recognized for antiradical, antimicrobial, and cytotoxic activities.[6,7] It is also known for hepatoprotective[8] and antidiabetic[9] potential.

Due to red in color, traditionally, beetroot is claimed to be useful in hematological disorders. However, no scientific evidence is available pertaining to it. Hence, the aim of the present work was to evaluate anti-anemic potential of beetroot.

EXPERIMENTAL

Plant collection

Beetroot *B. vulgaris* was collected from local market of Jabalpur in the month of March 2013. It was then identified and authenticated by Dr. A.B. Tiwari, Sr. Scientist,

Department of Corporation and Herbal Physiology, Jawaharlal Nehru Krishi Vishwavidyalaya, Jabalpur (Madhya Pradesh), India.

Extraction

B. vulgaris were washed sliced and shade dried under room temperature for a period of 2 weeks. The dried plant material was powdered and 100 g of powder was subjected to hot percolation in a soxhlet apparatus using ethanol. Extract was then dried and stored at 4°C till further use.

Phytochemical and phytoanalytical studies

Phytochemical tests

The extract was subjected to phytochemical analysis as per reported method.[10]

Estimation of folic acid

The standard or sample solution of folic acid 1.0 ml were mixed with 1.0 ml of 4 mol L-1 hydrochloric acid, 1.0 ml of 1% (w/v) sodium nitrite, 1.0 ml of 1% (w/v) sulfamic acid and 1.0 ml of 1% (w/v) 3-aminophenol, which was resulting in an orange-yellow complex. The absorption of complexation was measured at 460 nm using ultraviolet (UV)-visible spectrophotometer. A calibration curve was prepared at the range of 2-20 µg/ml and amount of folic acid in extract was estimated.[11]

Estimation of iron

A total of 100 mg of extract was transferred in 10 ml of water and transferred into a 100 ml volumetric flask, 5 ml of 2.058 mol/ml potassium thiocynate and 3 ml of 6 mol/ml nitric acid were added to develop the color. E volume was made to 100 cm^3 mark with de ionized water. Absorbance readings were measured for each including the standard solutions at 579 nm using UV-visible spectrophotometer.[12]

Estimation of vitamin C

Five grams of extract was homogenized with 25 ml of metaphosphoric acid-acetic acid solution, and it was quantitatively transferred into a 50 ml volumetric flask and shaken gently to homogenize solution. Later, it was diluted up to the mark by the meta phosphoric acid-acetic acid solution. The obtained solution is filtered and centrifuged at 4000 rpm for 15 min, 0.23 ml of 3% bromine water were added into 4 ml of centrifuged sample solution to oxidize the ascorbic acid to dehydroascorbic acid and after that 0.13 ml of 10 % thiourea to remove the excess of bromine. Then, 1 ml of 2, 4-dinitrophenylhydrazine solution was added to form osazone kept at 37°C temperature for 3 h in a thermostatic bath. After it was cooled in ice bath for 30 min and treated with 5 ml chilled 85 % H_2SO_4, with constant stirring. As a result, a colored solution's absorbance was taken at 521 nm. Concentration of ascorbic acid was determined using calibration of standard ascorbic acid (2-10 µg/ml).[13]

Acute toxicity studies and selection of dose

Jain and Singhai[14] reported the oral dose of 100 and 200 mg/kg of ethanol extract of beetroot for *in vivo* experiments determined as per organization for economic co-operation and development acute toxicity studies guidelines. Hence in the present work, same dose viz. 100 mg/kg and 200 mg/kg of extract was taken for studies.

Induction of experimental anemia

Anemia was induced by intraperitoneal injection of phenyl hydrazine (60 mg/kg, i.p., in divided doses daily, for 3 consecutive days.[15,16] Anemia was considered to be induced when red blood cell (RBC) level as well as hemoglobin concentration of the blood reduced by about 30%.

Study group design

After induction of anemia, rats were divided into groups, except Group I comprised of healthy rats. Grouping is as follows:

- Group I: Normal control
- Group II: No treatment
- Group II: Standard treatment (Ferritop-Z)
- Group III: Extract (100 mg/kg)
- Group IV: Extract (200 mg/kg).

Bio-analytical studies

The RBC number and hemoglobin concentration were determined every 3 days for 24 days.

Statistical analysis

Data are presented as mean ± standard error mean and analyzed by using one-way analysis of variance followed by Bonferroni's multiple comparison test (post-test); $P \leq 0.05$ was considered as statistically significant in all analyses.

RESULTS

Extraction and phytochemical screening

The yield of extract was 4.3 g. Extract was pale-brown in color. The results of preliminary phytochemical analysis

extract of *B. vulgaris* showed abundant presence of alkaloids, terpenoids, saponins, tannins, and polyphenols.

Phytoanalytical studies

Contents of phytoconstituents estimated are as follows:

- Folic acid: 2.21±0.54 µg/g of extract
- Ascorbic acid: 3.78±0.26 mg/g of extract
- Iron: 30.42±3.17 mg/g of extract.

Erythropoietic effect

Following the induction of anemia, the number of erythrocytes and the hemoglobin concentration decreased by 62.51% and 69.64%, respectively. Administration of standard hematinic preparation and extract (200 mg/kg) resulted in significant increase ($P < 0.001$; $P < 0.01$) in the number of RBCs as well as hemoglobin concentration when compared to the untreated phenylhydrazine-induced anemic rats (Figures 1 and 2).

DISCUSSION

Para substitution on phenyl rings is responsible for imparting nucleophillic character to phenyl hydrazine.[17] During the process of oxidation, it forms free radicals.[18] When enters into blood stream, it causes hemolysis, which arise due to oxidative alterations of blood cell proteins.[19] This process leads to premature aging of erythrocytes and predisposes to premature splenic sequestration. This leads to lack of circulating erythrocytes and hemoglobin.[20]

In the present work, anti-anemic activity of *B. vulgaris* was determined. Beetroot is rich source of folic acid, ascorbic acid, and iron. Folic acid/folate is a water soluble vitamin. It is found abundantly in green leafy vegetables, fruits, grains, and cereals.[21] A healthy person requires 200-400 µg folic acid per day, however, during pregnancy and lactation, its daily requirements become 500-600 µg.[22] Deficiency of folic acid in body may lead to pathogenesis of megaloblastic anemia and macrocytosis of erythrocytes.[23] Diet low in folic acid may increase risk of breast, pancreatic, and colon cancer.[24-26] Its low level in body may also predispose to coronary artery disease.[27] Ascorbic acid is an important component of human diet. It is an established fact that ascorbic acid is involved in release of iron from ferritin.[28] Reduced level of ascorbic acid and its decreased catabolism is also a condition observed during anemia.[29]

Diminution of levels of iron is one of the major hallmarks of anemia. According to a report of World Health Organization, about 35-75 % of pregnant women suffer from anemia.[30] This condition is observed when iron demand by body is more than its availability in body. This could be also due to insufficient iron intake, impairment in mechanism of iron absorption and transportation and/or chronic blood loss.[31]

In this work, reference hematinic preparation (Ferritop-Z) contains iron that forms an integral part of hemoglobin. Various vitamins from B vitamin family are responsible to a precursor in creation of cofactors for hematopoiesis. Extract effectively raised the level of hemoglobin

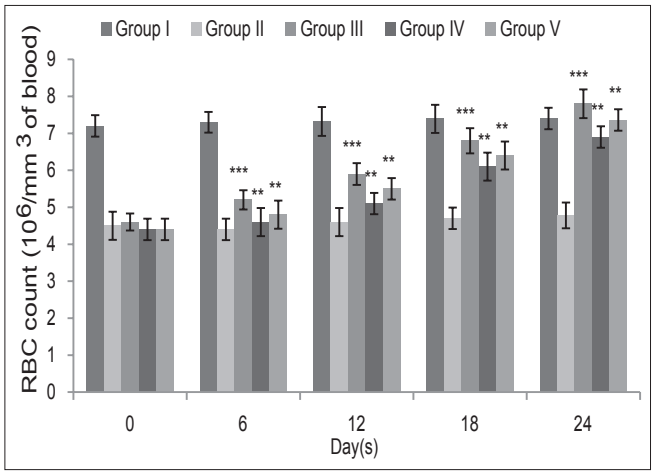

Figure 1: Effect of *Beta vulgaris* extract on red blood cell count in phenylhydrazine induced anemic rats. Data are presented as mean ± standard error mean and analyzed by one-way analysis of variance followed by Bonferroni's multiple comparison test (post-test); $P \leq 0.05$ was considered statistically significant in all analyses.

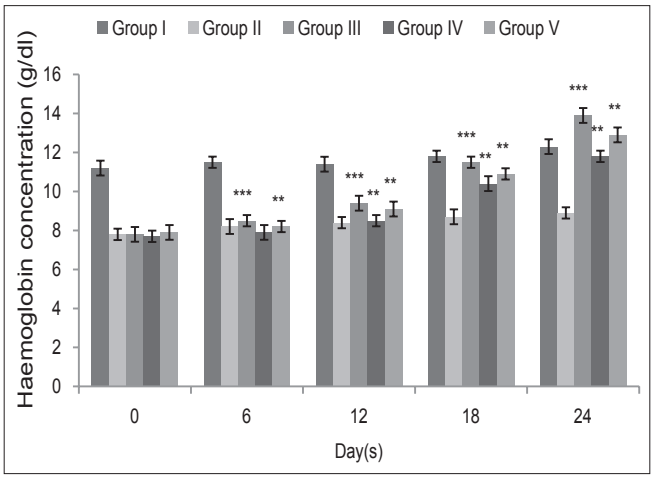

Figure 2: Effect of *Beta vulgaris* extract on hemoglobin concentration in phenyl hydrazine induced anemic rats. Data are presented as mean ± standard error mean and analyzed by one-way analysis of variance, followed by Bonferroni's multiple comparison test (post-test); $P \leq 0.05$ was considered as statistically significant in all analyses.

and erythrocyte count (at dose 200 mg/kg). Vitamin and minerals found in beetroot are most likely active ingredients responsible for its hematinic effects. Still, methodical studies are obligatory to derive its effects on humans.

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Comparative Studies on Antioxidant Activity, Total Phenol Content and High Performance Thin Layer Chromatography Analysis of Seabuckthorn (*Hippophae rhamnoides* L) Leaves

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ABSTRACT

Background: Seabuckthorn (SBT) is a high altitude medicinal plant with vast history of use in traditional medicinal systems such as Tibetan and Chinese systems. SBT leaves have shown range of pharmacological properties suggesting their importance to be used for product development. **Objective:** The aim of this study was to compare 75% ethanolic extracts of male and female SBT leaves on the basis of antioxidant activity, total phenol content and *high performance thin layer chromatography* (HPTLC) estimation of β-sitosterol and ursolic acid. It also involved comparison of total phenol contents of successive soxhlet extracts (pet ether, chloroform, ethyl acetate, ethanol, and aqueous) of above leaves. **Materials and Methods:** Antioxidant activities and total phenol contents of the extracts were evaluated by using 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging assay and Folin–Ciocalteu reagent based assay, respectively. **Results:** Male leaf extract was found to show significantly higher antioxidant activity and total phenol content than that of female leaves. Furthermore, the successive extracts of male leaves showed higher phenol contents than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts. In HPTLC estimation, concentration of β-sitosterol in female leaf extract was observed to be less than that of male leaf extract. However, ursolic acid concentration was found to be almost same in both the type of leaf extracts. **Conclusion:** The results suggest the need for developing standard quality control profile of SBT leaves, especially for product development.

Keywords: Antioxidant activity, 75% ethanolic extract, high performance thin layer chromatography, seabuckthorn, total phenol content

INTRODUCTION

Free radical formation inside the body is a natural and unavoidable phenomenon. Free radicals cause damage to various biomolecules such as lipid, protein, and DNA of the living systems.[1] There are two interdependent systems in the body, oxidants and antioxidants. Increase in the amount of free radicals shifts the balance toward oxidants resulting in oxidative stress.[2] Plant derived antioxidants have the potential to reduce the incidence of oxidative stress by shifting the balance towards antioxidants.[3] There are reports of increased interest on plant based antioxidants.[4,5]

Hippophae rhamnoides L., commonly known as seabuckthorn (SBT); Family: Elaeagnaceae, growing in North-West Himalayas at high altitude (7,000-15,000 feet), is a dwarf to tall (3-15 feet), branched, and thorny nitrogen fixing deciduous shrub, native to Europe and Asia.[6] SBT is a good source of a large number of nutrients and phytochemicals, especially phenolic compounds.[6-9] Guliyev *et al.* (2004) have reported that the phenolic acids found in the leaves of SBT include gallic, protecatechuic, p-coumaric, ferulic, p-hydroxybenzoic and ellagic acids.[10] Suryakumar *et al.* (2011) have reported high contents of ursolic acid in SBT.[11]

SBT leaf extracts have been reported to possess many medicinal properties, including antioxidant.[6,12-15] Medicinal effects of SBT are due to the presence of high antioxidant

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contents.[6] There may be some difference between antioxidant properties and phytochemical contents of male and female SBT leaves. Furthermore, no such studies have been found comparing male and female SBT leaves on the basis of these parameters. In view of the above, the present study was undertaken to compare 75% ethanolic extracts of male and female SBT leaves on the basis of antioxidant activities, total phenol contents and *high performance thin layer chromatography* (HPTLC) estimation of β-sitosterol and ursolic acid contents.

MATERIALS AND METHODS

Plant material

Leaves of SBT were collected from Leh (Ladakh), India, and authenticated by National Institute of Science Communication And Information Resources, New Delhi, India.

Chemicals

Gallic acid, 2,2-di phenyl-1-picryl hydrazyl (DPPH), Folin–Ciocalteu reagent (FCR) were purchased from sigma. All other reagents were of analytical grade and were procured from Ranbaxy Fine Chemicals Ltd., Punjab, Fischer Inorganics and Aromatics Ltd., Madras, NICE Chemicals Ltd., Cochin and Central Drug House Pvt., Ltd., New Delhi.

Preparation of extracts

75% ethanolic extract

Powdered SBT leaves were extracted with 75% ethanol by cold percolation method.[6] The powdered leaves were soaked in above solvent (1:8 w/v) at room temperature. After 24 h, the supernatant was decanted and the residue was re-soaked in fresh solvent. This process was repeated 5 times for complete extraction. After completion of the extraction process, the supernatants were pooled and filtered through 250 mesh nylon cloth. This filtrate was dried under reduced pressure until a solid mass was obtained. Same procedure was repeated for, both male and female leaves. The above extracts were used for estimation of total phenol contents, antioxidant activities and for HPTLC estimation of β-sitosterol and ursolic acid.

Successive extracts

Powdered SBT leaves were extracted, one by one, with petroleum ether (50-60°C), chloroform, ethyl acetate, ethanol and distilled water, by soxhlet extraction process for 4 h. After completion of extraction process, solvents were removed by distillation and concentrated under reduced pressure till a solid mass was obtained. Same procedure was repeated for, both male and female leaves. The above extracts were used for estimation of total phenol contents.

Total phenol content

Total phenol content was estimated in above prepared SBT extracts by FCR based assay.[16] To the aliquot (50 µl) taken from stock solution (1 mg/ml) of the extract, 3.5 ml distilled water and 250 µl of FCR was added, the mixture was kept at room temperature for 1-8 min and 750 µl of 20% sodium carbonate solution was added. Mixture was kept at room temperature for 2 h and absorbance of the color developed was recorded at 765 nm with the help of a ultraviolet (UV)-visible spectrophotometer against blank. Total phenolic content was determined using gallic acid standard curve (R^2 = 0.986) and expressed in mg/g as gallic acid equivalents. Same procedure was repeated for estimation of total phenolic content in all the extracts.

Antioxidant activity

DPPH based assay was used for determination of antioxidant activities of above prepared extracts.[17] A 0.1 mm solution of DPPH was prepared by using methanol. A volume of 2 ml of this solution was added to 2 ml of solution of extract and the mixture was kept in dark for 20 min. After 20 min. absorbance of the color developed was recorded at 517 nm with the help of a UV-visible spectrophotometer against blank. Control was prepared by adding 2 ml of the DPPH solution to 2 ml methanol. Same procedure was repeated for estimation of antioxidant activity in all the above extracts.

IC50 values were calculated using the formula:

Percentage Scavenging = (Absorbance [Control-Test]/ Absorbance of Control) × 100

HPTLC analysis

This was perfomred by using 2 different reference standards, namely β-sitosterol and ursolic acid. A stock solution was prepared for each reference standard i.e. β-sitosterol and ursolic acid (1 mg/ml) and sample that is., 75% ethanolic extract of SBT leaves, both male and female (40 mg/ml). Firs, the TLC plates (precoated silica gel 60-F254) were kept at 110°C for 1 h for activation, followed by the test sample and reference solutions (5 µl each with band length of 8 mm) were applied on the TLC plates through

CAMAG linomat-5 applicator. After drying, the plates were developed in CAMAG twin trough TLC chambers using solvent system toluene: Ethyl acetate (8:2, saturation time 5-10 min). The plate was properly dried in air and was sprayed with anisaldehyde-sulphuric acid solution, followed by heating at 110°C, and scanned through CAMAG TLC Scanner 3 at 365 nm via UV (D2 Lamp) with the help of WinCATS (1.3.2.0) software. Same procedure was used for both male and female leaf extracts.

Statistical analysis

All the analyses, except HPTLC analysis, were carried out in triplicate and the results were expressed as mean ± standard deviation. Statistical analysis was performed using Statistical Package for the Social Sciences (12.0) Software.

RESULTS AND DISCUSSION

Total phenol content and antioxidant activity

Table 1 represents the total phenol content and IC_{50} values (antioxidant activities) of 75% ethanolic extracts of male and female SBT leaves. This table showed that total phenol content of 75% ethanolic male leaf extract was much higher than that of female leaf extract. Also, the IC_{50} value of male leaf extract was significantly lower than that of female leaf extract, which indicate that antioxidant activity of male leaf extract is significantly higher than that of female leaf extract.

On comparing the total phenol contents and antioxidant activities of 75% ethanolic extracts of both, male and female SBT leaves, an increase in antioxidant activity with increase in total phenol content was observed. This observation indicates that phenolic compounds may contribute toward antioxidant activity observed (Table 1). Further, it also supports the work of other researchers that phenolic compounds are known to show various properties including antioxidant property.[5,18,19] Also, the beneficial effects of natural phenolic compounds of plant origin, on coronary heart diseases and cancers have been reported to be mainly due to their antioxidant activity.[5] Considering these points, the significant difference in total phenol contents of 75% ethanolic extracts of male and female SBT leaves may lead to significant variation in amounts of pharmacological effects precipitated by these extracts. Hence, one should be careful while collecting the SBT leaves especially for use in product development because variation in amount of pharmacological effects of the extract may result in variation in medicinal effects of the finished products also, which may cause batch to batch consistency problems. These observations suggest the need of quality control of SBT leaves.

Table 1: Total phenol content and IC_{50} values of 75% ethanolic extracts

75% ethanolic extracts	Total phenol content (GAE mg/g)	IC_{50} For antioxidant activity (µg/ml)
Male leaf	398.86±2.20*	5.99±0.25*
Female leaf	134.95±2.72*	30.39±0.23*

*Significant difference (P<0.01). GAE: Gallic acid equivalent

Table 2 represents total phenol content of successive extracts of male and female SBT leaves. It revealed that, even in successive extracts, total phenol contents of male leaf extracts were more than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts. Both the leaves were found to show maximum content of total phenols in aqueous extracts, which indicate that large quantity of phenolic compounds, present in both male and female SBT leaves, are hydrophilic (polar) in nature. However, they appear to be present in more quantity in male leaves since total phenol content in aqueous extract of male leaves was slightly higher than that of female leaves (Table 2).

In case of ethyl acetate and ethanol extracts also, total phenol contents of male leaves was much higher than that of female SBT leaves, which suggests that phenolic compounds with hydrophobic (non-polar) nature are present in significantly lower amounts in female SBT leaves than in male leaves (Table 2). These findings support the lower content of total phenols in 75% ethanolic extract of female leaves (Table 1) since ethanol is a non-polar solvent.

HPTLC analysis

It involved simultaneous estimation of β-sitosterol and ursolic acid in the 75% ethanolic extracts of both, male and female SBT leaves.

Table 3 represents the results of HPTLC analysis. It stated that concentration of ursolic acid was almost same in case of both the type of leaf extracts but concentration of β-sitosterol was higher in male leaf extract than that of female.

Table 2: Total phenol content of successive extracts of male and female SBT leaves

Successive extracts	Total phenol content (GAE mg/g)	
	Male leaf	Female leaf
Pet ether	63.45±2.10**	62.18±1.95**
Chloroform	52.17±1.73**	51.68±2.41**
Ethyl acetate	146.29±3.12*	75.90±3.03*
Ethanolic	432.19±2.56*	84.190±3.67*
Aqueous	599.67±3.60*	587.33±2.83*

*Significant difference; **Non significant difference, GAE: Gallic acid equivalent, SBT: Seabuckthorn

Table 3: HPTLC analysis

Analytes	R_f	Male leaf extract (% w/w)	Female leaf extract (% w/w)
β-sitosterol	0.52	1.77	0.03
Ursolic acid	0.34	0.265	0.260

Above results can be summarized as follows.

a. 75% ethanolic male leaf extract was observed to have significantly higher total phenol content and antioxidant activity (DPPH scavenging activity) than that of female leaf extract.
b. Increased antioxidant activity of 75% ethanolic male leaf extract may be due to the presence of higher amount of total phenol content. However, it needs to be investigated further.
c. Even successive extracts of male leaves was observed to show higher quantities of total phenols than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts.
d. Concentration of β-sitosterol in female leaf extract was observed to be less than that of male leaf extract. However, ursolic acid concentration was found to be almost same in both the type of leaf extracts.

CONCLUSION

The results of the present investigation demonstrate some important differences between 75% ethanolic extracts of male and female SBT leaves with respect to total phenol content, antioxidant activity and HPTLC estimation of β-sitosterol and ursolic acid contents. Results also indicate the need for quality control of SBT leaves, especially for product development.

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Hepatoprotective Effect of *Calotropis procera* in Isoniazid and Rifampicin Induced Hepatotoxicity

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ABSTRACT

Objective: In this study anti-tubercular (Anti-TB) drugs (isoniazid [INH] and rifampicin [RMP]) induced liver toxicity has been studied for the hepatoprotective effect of hydroethanolic extract of *Calotropis procera* (CP) flowers in rats. **Materials and Methods:** Animals were divided into four groups, group A was given normal saline (1 ml/kg), group B received INH (50 mg/kg) and RMP (100 mg/kg) group C received INH (50 mg/kg), RMP (100 mg/kg) and CP (150 mg/kg) orally for 14 days. **Results:** Biochemical markers of liver toxicity such as aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and bilirubin and tissue histology were done inall groups. Anti-TB drugs (INH 50 mg/kg and RMP 100 mg/kg) have enhanced the ALT, AST, ALP, bilirubin and histological changes in liver, whereas co-administration of anti-TB drugs with CP has reduced these levels within the normal range. **Conclusion:** Findings of this study showed the hepatoprotective effect of CP against INH and RMP administration to reduce the liver damage for chronic treatment.

Keywords: *Calotropis procera*, hepatoprotective, isoniazid, rifampicin

INTRODUCTION

After human immune deficiency virus infection also known as acquired immune deficiency syndrome, tuberculosis (TB) has the highest mortality rate in the world.

A 2012 report of World Health Organization revealed that in 2011, 8.7 million people fell ill with TB and 1.4 million deceased from TB.[1] If active TB remained untreated or treatment is interrupted, two out of every three patients died, which deemed it necessary to carry out instantaneous, most suitable and uninterrupted treatment for this disease.[2] Hepatotoxicity is one of the most serious adverse drug reactions of anti-tubercular (anti-TB) treatment (ATT), which limits the use of these drugs.[3]

Anti-TB drugs induced hepatotoxicity is the condition in which liver enzymes aspartate aminotransferase (AST) and alanine transaminase (ALT) increased 3-5 times of normal levels, in severely damaged liver it can cause uncontrolled hepatitis, and 1-2% patients stop the treatment.[4]

A mortality rate of 5% is reported worldwide due to anti-TB drug-induced hepatotoxicity.[5]

One of the most important first-line anti-TB drug regimens included rifampicin (RMP), pyrazinamide and isoniazid (INH), which can induce hepatic injury. The co-administration of RMP and INH is known to increase the risk of hepatic injury.[6] In slow metabolizers RMP can increase the hydrazine production induced by INH hydrolase when given concurrently with INH. This can explain the increased hepatotoxicity of these drugs in combination when compared to their individual administration.[7]

Hepatotoxicity caused by anti-TB drugs can affect the hepatocytes or vasculature and the biliary epithelium, but the exact underlying mechanism and contributing factors causing hepatic damage are not clearly known. Temporary and asymptomatic increased ALT levels may indicate minor and non-progressive damage to mitochondria, cell membranes, or other structures of hepatocytes.[8] Infrequently these changes leads to inflammation, cell death, or major histopathological changes.[9] On histopathological examination, focal hepatic necrosis maybe revealed with bridging in serious cases.[10]

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Various studies have been conducted to develop the most efficient way to eliminate or minimize the hepatotoxicity of anti-TB drugs, by using the natural botanical and/or synthetic drug products without interacting their therapeutic actions.[11]

It has been observed that the antioxidant action is the common mechanism of various herbal drugs.[12,13] Natural plant products are extensively use in different disease conditions and serve as compounds of interest both in their natural form and for the synthetic derivatization.

The importance of natural products in current medicine has been acknowledged.

Approximately, more than 20 new drug products introduced world over between 2000 and 2005, are derivatives of natural products.[14] It has been documented that natural products and related drugs are used to treat 87% of all categorized disease indications in humans.[15] Among the herbal drugs, *Calotropis procera* (CP), which is a wild growing plant also possess flavonoids, alkaloids, cardiac glycoside, stanins, sterol, and triterpines. The flowers are reported to contain flavonoids, querectin-3-rutinoside, sterols, etc. Flavonoids are reported to possess anti-oxidant and hepatoprotective properties.[16]

CP, a wild growing plant belongs to *Asclepiadaceae* family.[17] Its different parts exhibit antioxidant, analgesic, and anti-inflammatory properties.[18] The current study was carried out to evaluate protective effects of the flowers of CP using anti-TB drugs induced hepatic toxicity model in rats.

MATERIALS AND METHODS

Research design and setting

This was an experimental study which was conducted in Dow International Medical College, Department of Pharmacology and Therapeutics in association with animal house and Dow Diagnostic Research and Reference Laboratory.

Animals

Adult Wistar rats (185-195 g) of either sex were obtained from the Charles's River Breeding Laboratory USA, cross breaded in the Dow University Animal House.

All animals were acclimatized prior to the experiment in standard environmental conditions and given the rodent diet with water ad libitum for 1 week. Approval was obtained from the Institute of Basic Medical Sciences, Institutional Review Board, Funding Committee and finally from the Board of Advance Studies and Research of Dow University of Health Sciences.

Plant

The flowers of CP were collected in 2nd week of August from the vicinity of Dow University Ojha Campus Karachi. Dow College of Pharmacy, Pharmacognosy Department recognized and a specimen was deposited in the museum with No: DCP/H/00427 for herbarium.

Preparation of the flowers extract

The flowers were dried in the shade and were macerated with 70% ethanol and 30% water, and extract was dried out for concentrate at 40°C under vacuum by using a Rota evaporator, which gave 25% of the extract.

Drug

INH (100 mg/tablet) and RMP (150 mg/tablet) tablets were used. Tablets were dissolved in the distilled water, and doses were calculated according to the body weight.

Hepatoprotective activity

The method of Lenaerts *et al.*[19] was used in the study. Animals were divided into three groups of six animal of each and drugs were administered from the oral route with the help of stainless steel feeding needle for rodents for 2 weeks. Group A was treated as control group, which received normal saline solution 1 ml/kg. Group B was received INH 50 mg/kg and RMP 100 mg/kg for to induce hepatotoxicity. Group C received INH 50 mg/kg RMP 100 mg/kg and CP extract 150 mg/kg taken as anti-TB drugs plus CP treated group. All animals were sacrificed after giving deep anesthesia of chloroform. Blood was collected for the liver function tests after cardiac puncture and liver was removed for its histopathological examination, which was conducted at Dow Diagnostic and Research Laboratory.

Histology

Hematoxylin and eosin stain technique is used to stain the histopathology slides. The slides were examined under light microscope for general architecture of hepatic parenchyma (intact/disturbed), portal area (normal/disturbed), central vein (normal/disturb), hepatocytes (cytoplasm and nucleus status), sinusoids (normal/dilated), fibrosis (present/absent), necrosis (present/absent), cholestasis

(present/absent), any other findings (granulomas, etc.).[20] Microscopic findings of inflammation were converted into numerical data using the Knodell score or histologic activity index (Table 1).[21]

Biochemical parameters

The blood samples were obtained for the analysis of various biochemical parameters, which include the estimation of serum alanine ALT, AST,[22] alkaline phosphatase (ALP),[23] total bilirubin,[24] and direct bilirubin[25] levels.

Statistical analysis

The data were expressed as mean ± standard error of the mean, (n = 6) and statistical analysis was performed by using the Statistical Package for Social Science program version 16 by IBM. Data were analyzed using one-way analysis of variance, followed by multiple comparisons using Dunnett's procedure to compare all groups against Group-A. $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Anti-TB drugs INH 50 mg/kg and RMP 100 mg/kg (Group B) have increased the levels of ALT, AST, ALP bilirubin (total and direct), and microscopic finding of inflammation in the liver when compared with the control (Group A). Administration of CP (70% hydroethanolic extract) in doses of 150 mg/kg along with the INH and RMP significantly hindered the rise in the levels of ALT (t = 3.6872, P = 0.0042), AST (t = 3.947, P = 0.0027), ALP (t = 3.2909, P = 0.0081), total bilirubin (t = 2.789, P = 0.0191), direct bilirubin (t = 1.664, df = 10, P = 0.1271) and microscopic inflammatory findings (t = 3.9936, P = 0.0025) when compared with the Group B. Values are mentioned in Table 2.

Table 1: Knodell scoring system for portal inflammation

Portal inflammation	Score
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4

Anti-TB drugs, especially INH and RMP are used in combination, which is significantly hepatotoxic and it is documented in earlier studies.[26,27]

The mechanism of hepatotoxicity has been suggested by these two drugs is mediated via oxidative damage,[28] the additive or synergistic hepatotoxic effect has been reported to the caused by monoacetyl hydrazine, hydrazine and other related compounds produced from hepatic biotransformation through enzyme induction;[29] therefore, a regular weekly or biweekly monitoring of liver enzyme is required for initial 2 months,[30] according to the guidelines of American thoracic society a rapid increase in liver enzyme like ALT is one of the most prominent indicator for development of hepatic injury.[31,32] In order to manage the ATT induced hepatotoxicity it is recommended to hold the treatment when the hepatotoxicity is evident, until the liver enzymes normalize.[33,34] Alternatively co-administration of appropriate hepatoprotective agents would prevent this hepatotoxicity.[35] Literature survey revealed that, the CP is one of the many plants have the potential to be used for hepatoprotective effects against the drug or chemical-induced hepatotoxicity.[36,37] CP is one of the plants used to protect the liver injury. Flavonoids are the active constituents of the flower of CP act as antioxidants against the reactive oxygen moieties by boosting the endogenous scavenging system of the body, these are also known to interfere various free-radical producing systems and also enhance the endogenous antioxidant function.[38] Flavonoids are oxidized by radical, which makes a more stable and less reactive free radicals and made the radicals inactive.[39] Another mechanism of flavonoids as antioxidants is to prevent the reaction of free radicals with nitric oxide

Table 2: Effects of CP floral extract on enzymes and histology of liver in anti-TB drug-induced hepatic toxicity in rats

	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total bilirubin mg/dl	Direct bilirubin mg/dl	Microscopic finding	One-way ANOVA
Group A control (saline 1 ml/kg p. o. 2 weeks)	20.16±4.49	133.25±10.2	74.00±7.2	0.22±0.05	0.06±0.02	17.75±3.21	df=5-12 F=15.564 *P*<0.01
Group B anti-TB drugs (INH 50 mg/kg+RMP 100 mg/kg p. o. 2 weeks)	41.83±7.09	189.92±17.3	154.67±18.5	0.61±0.07	0.17±0.03	41.66±4.61	
Group C treatment (INH 50 mg/kg+RMP 100 mg/kg+ CP150 mg/kg p. o. 2 weeks)	13.83±2.72*	113.5±8.7*	83.33±11.3*	0.37±0.03**	0.11±0.02^{NS}	18.15±3.82*	

n=6. **P*<0.01, ***P*<0.05 versus anti-TB drugs. NS: Non-significant *post-hoc t*-test, INH: Isoniazid, RMP's: Rifampicin, CP: *Calotropis procera*, ALT: Alanine transaminase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, ANOVA: Analysis of variance, anti-TB: Anti-tubercular

causing less damage.[40] Flavonoids are also found to inhibit xanthine oxidase activity, which is a source of free radicals leads to the oxidative injury to the tissues.

A further mechanism of flavonoids to decrease the number of immobilized leukocytes to endothelial wall causing derivation of oxygen-derived free radicals and cytotoxic oxidants and inflammatory mediator preventing the inflammatory condition.[41]

Degranulation of neutrophils in plasma membrane by modulating Ca^{2+} channels ban be hampered by flavonoids.[42]

Various studies proved the successful use of rat's models for INH and RMP induced hepatotoxicity.[11,43] Therefore, same model has been used to determine the hepatoprotective activity of CP in the anti-TB drug-induced toxicity. INH and RMP were given daily for 14 days to produce liver toxicity at very high doses (INH 50 mg/kg, RMP 100 mg/kg) as compared with humans because rats metabolize drugs faster than humans and period of study shorter in comparison to the treatment of TB in humans.[44] At day 15th after the last dose administration hepatic injury was assessed by the measurement of liver enzymes (ALT, AST, ALP, and Bilirubin) and the presence of microscopic findings.

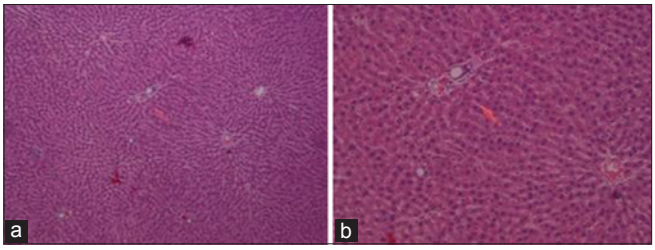

Figure 1: Photomicrograph of control group showing no inflammation around the portal triad (H and E, ×10) (a) normal view, (b) enlarged view.

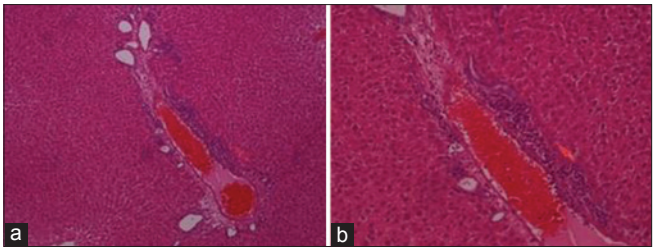

Figure 2: Photomicrograph of anti-tubercular treated group showing inflammation around the portal triad (H and E, ×10) (a) normal view, (b) enlarged view.

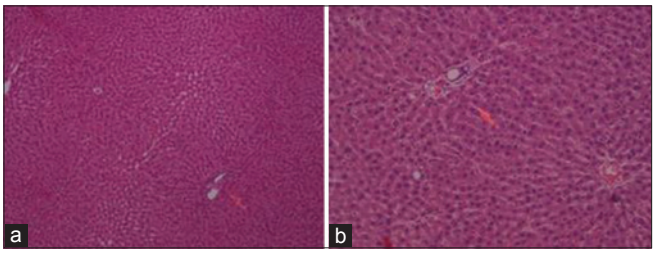

Figure 3: Photomicrograph of anti-tubercular plus calotropis group showing no inflammation around the portal triad (H and E, ×10) (a) normal view, (b) enlarged view.

In the current study, rats were divided into three groups in which Group A received the normal saline as control and Group B received the anti-TB drugs, while Group C received the anti-TB drugs along with the CP extract.

On the comparison of Group A with Group B, there were significant increased ($P < 0.05$) in the liver enzyme levels, and microscopic findings shown in Table 2, Figures 1 and 2, which were in agreement with previous studies, furthermore when Group B was compared with the CP treated Group C, the levels of enzymes and microscopic findings were significantly reduced and brought to the normal range shown in Table 2 and Figure 3. Therefore, these changes suggested that the extract of CP flowers possess hepatoprotective activity, which was further evident by the significant ($P < 0.01$) reduction in inflammatory changes in liver tissues. These finding completely favor the previously conducted studies in which co-administration of CP showed hepatoprotective effect against hepatotoxic drugs and demands the further experiments on higher animals to prove its safe and effective use to reduce the liver damage with such toxic drugs for chronic treatment.[16,45-47]

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Antihyperglycemic Activity and Standardization of the Bioactive Extract of *Cleome droserifolia* Growing in Egypt

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ABSTRACT

Background: *Cleome droserifolia* herb is well known in the Egyptian folk medicine for the treatment of diabetes. However, a standardized active extract of the herb was never prepared for incorporation into a pharmaceutical dosage form. **Materials and Methods:** Comparative high performance liquid chromatography (HPLC) chromatographic profiles were established in order to study the ethnopharmacological use of the antihyperglycemic herb using a validated reversed phase-HPLC method which was developed for standardization of the active aqueous extract. A biologically guided fractionation of the antihyperglycemic aqueous extract was carried out *in vivo* using alloxan induced diabetic rats. **Results:** The aqueous extract contained the highest percent of the total active flavonol glycosides (78.20%) compared to the 70% and 50% ethanolic extracts (51.17 and 42.66%, respectively). The aqueous extract and its ethyl acetate fraction possessed the highest antihyperglycemic activities. A standard calibration curve, established for the major bioactive methoxylated flavonol glycoside (kaempferol-4′-methoxy-3,7-dirhamnoside) at a concentration range of 44-174 µg/ml, showed good linearity with a correlation coefficient (R2) of 0.998. The recovery of the method was 100.5%. A high degree of precision (relative standard deviation values <5%) was achieved. The limits of detection and quantification were 0.01 and 0.02 µg/ml, respectively, indicating the sensitivity of the method.**Conclusion:** The aqueous extract contained the highest percent of the total active flavonol glycosides. The extract, standardized to contain not <1.5 ± 0.06% of kaempferol-4′-methoxy-3,7-dirhamnoside, was tested at three different dose levels showing a 63.3% activity of that of metformin at100 mg/kg body weight. Furthermore, it raised the blood insulin level by 146.26% at this dose level.

Keywords: Antihyperglycemic, *Cleome droserifolia,* high performance liquid chromatography standardization, kaempferol-4′-methoxy-3,7-dirhamnoside, validation

INTRODUCTION

The dried herb of *Cleome droserifolia* (Forssk.) Del, a plant of the Cleomaceae family,[1] locally known as Samwah is well known in the Egyptian folk medicine as its decoction has been used by the Bedouins of the southern Sinai for the treatment of diabetes.[2] The herb is also sold by herbalists in the Egyptian market as an antihyperglycemic agent. Several articles discussed the efficacy and safety of *C. droserifolia*.[2-7]

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Nevertheless, a standardized extract of the herb was never prepared nor incorporated into a pharmaceutical preparation for the treatment of diabetes.

In a previous work of our group, cell-based bioassays were applied to determine active compounds, which could be used as bioactive markers to standardize the herbal extract of *C. droserifolia* and hence assure its quality and efficacy.[8,9] The aqueous extract of *C. droserifolia* and its ethyl acetate fraction showed significant insulino-mimetic effects in peripheral tissues. We isolated three flavonol glycosides; isorhamnetin-3-O-β-D-glucoside (F1), quercetin-3'-methoxy-3-O-(4'-acetylrhamnoside)-7-O-α-rhamnoside (F2) and kaempferol-4'-methoxy-3,7-dirhamnoside (F3) from the ethyl acetate fraction. These compounds proved to possess a high antihyperglycemic activity, which was twice as active as insulin in stimulating glucose uptake in differentiated C2C12 skeletal muscle cells.[8,9]

The aim of the present study was to support our previous *in vitro* results by *in vivo* experiments, compare two hydroalcoholic extracts of *C. droserifolia* to the aqueous extract regarding the active flavonol glycoside content, and prepare a standardized active extract through a validated reversed phase-high performance liquid chromatography (RP-HPLC) method using the major bioactive methoxylated flavonoid glycoside. Kaempferol-4'-methoxy-3,7-dirhamnoside (F3) was selected as a marker for HPLC standardization as it was the major bioactive compound we previously isolated from the ethyl acetate fraction.

MATERIALS AND METHODS

Plant material

The aerial parts of *C. droserifolia* were obtained from the Medicinal Plants Society, Saint Catherine, Sinai in 2008 and 2009. The plant was authenticated by Assistant Prof. Dr. M. Gebali (Plant Taxonomy and Egyptian Flora Department, National Research Center, Giza, Egypt). A voucher specimen (no. 313) was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Preparation of extracts and fractions

For comparative HPLC analysis of the extracts, 50 g of the air-dried aerial parts of *C. droserifolia* were extracted with water, 50% and 70% ethanol in water (2 × 500 ml) on cold, each time for 3 days and the volume was adjusted to 1 L in a volumetric flask.

The aqueous extract of the air-dried aerial parts of *C. droserifolia* as well as its fractions; the n-hexane, chloroform, ethyl acetate and *n*-butanol fractions were prepared as mentioned in Ezzat and Abdel Motaal, 2012.[8]

Chemicals

Alloxan was purchased from Sigma-Aldrich (St. Louis, MO, USA). Carboxymethyl cellulose sodium (CMC-Na) was purchased from Acros Organics (NJ, USA), while heparin sodium was purchased from Merck (Dramstadt, Germany). Insulin kit (Coat-A-Count Insulin) was purchased from Siemens, Medical Solutions Diagnostics (LA, USA). All reagents for extraction were of analytical grade (ADWIC, Egypt). Metformin, CID Co., Egypt. Chromatographic grade-double distilled water; analytical grade *O*-phosphoric acid and HPLC grade methanol were purchased from Merck, Germany. The acidic aqueous solution used for HPLC analysis was filtered through Agilent Ecno 0.45 µm polytetrafluoroethylene membrane filter and degassed in an ultrasonic bath before use. The marker flavonoid, kaempferol-4'-methoxy-3,7-dirhamnoside (F3), was previously isolated and identified by the authors.[8]

Animals

Adult male rats of Sprague-Dawley strain (130-150 g body weight) were obtained from the animal house of the National Research Center, Giza, Egypt. They were kept under the same hygienic conditions and were fed by the basal diet recommended by the American Institute of Nutrition.[10] Animals were housed for 2 days under standard conditions (well ventilated, temperature 22 ± 2°C, relative humidity 50-60% and 12 h day and night cycle). Food consisted of normal rat chow and water was provided *ad libitum*. Care was taken to avoid stressful conditions. All experimental procedures were performed between 8:00 and 10:00 a.m. All the animal experimental work was carried out after the approval of the Institutional Animal Ethical Committee.

RP-HPLC analysis of the extracts and fractions

An HPLC method was developed for the comparative study of the different extracts of *C. droserifolia* namely the aqueous, 50% and 70% extracts in order to reach the best solvent for extraction of the active flavonol glycosides. An Agilent technologies 1100 series HPLC was used, equipped with an Agilent 1200 series G1322A quaternary pump and degasser, a G1314A variable wavelength detector and an Agilent ChemStation software, Santa clara, California, United States software. Samples (1 mg/ml methanol) were injected into a lichrosphere 100 RP-18, 5 µm, 250 mm × 4 mm column (Merck, Germany) maintained at a temperature of 25°C, guarded by a 5 µm, 10 mm × 4 mm guard column. The mobile phase used was acetonitrile (solvent A) and 0.3% *O*-phosphoric acid in water (solvent B). A continuous gradient elution (10-75% A in B) for 25 min was carried out at a flow rate of 1.0 ml/min. The injection volume was 20 µl and detection was made at 325 nm. The same RP-HPLC method was used for the analysis of the chloroform, ethyl acetate and *n*-butanol fractions (1 mg/ml methanol, each).

Screening for *in vivo* antihyperglycemic activity

The 70% ethanolic and aqueous extracts of the aerial parts of *C. droserifolia* were assessed for their antihyperglycemic activity in rats over 28 days. In addition, the *n*-hexane, chloroform, ethyl acetate and *n*-butanol fractions of the aqueous extract were tested. All samples were tested at the same dose level of the biguanide metformin (150 mg/kg body weight). Diabetes was induced intraperitoneally in

the rats with a single dose of alloxan (150 mg/kg body weight).[11] The antihyperglycemic activity of the most active aqueous extract of the aerial parts of *C. droserifolia* was assessed in a further experiment over 28 days using three different dose levels; 50, 75 and 100 mg/kg body weight.

The control diabetic group in each experiment received a single daily dose of 1% CMC-Na. The vehicle, metformin and plant extracts were given orally by gavage as single daily treatments for 4 weeks. Blood samples in each experiment were collected from tail vein and fasting blood glucose levels of the overnight fasted animals at 0 time, and at days 14 and 28 from the treatment.

Estimation of plasma insulin level

At the end of the 28th day in the second experiment, 3 h after the last dose of the vehicle or the aqueous extract, blood samples were withdrawn from the orbital sinus of rats under light ether anesthesia into heparinized tubes. Samples were centrifuged at 3500 g for 15 min for separation of plasma. Plasma samples were separated and kept at −20°C for analysis when required. Insulin concentrations were determined by radio immunoassay procedure using insulin kit.

Standardization of the aqueous extract using RP-HPLC

Sample preparation

The aqueous extract (20 mg) was dissolved in 1 ml of 40% methanol in H_2O, using an ultrasonic, and loaded on an EX trelut® prepacked column (Merck). Elution was carried out using 7 × 1 ml, 40% methanol in H_2O, and the volume completed to 10 ml in a volumetric flask.

Construction of the standard calibration curve

Serial dilutions of F3 (50, 100, 120, 160, 200 μg/ml) were prepared from a stock solution having a final concentration of 200 μg/ml, formed by weighing accurately 5 mg in a 25 ml volumetric flask and dissolving in methanol. The purity of the standard F3 used was 87%, where the area-percent method was used to correct for the purity. A standard calibration curve was established using the corrected concentrations (44, 87, 105, 139 and 174 μg/ml). Each sample was injected in triplicates.

Validation of the RP-HPLC method

Linearity was determined by injecting five different concentrations of F3 standard solution (44-174 μg/ml). The accuracy was calculated as the percent recovery of spiked aqueous extract samples with F3 sample at a concentration of 139 μg/ml. To determine the intra- and inter-day precision of the method, F3 was assayed at two different concentrations (44 and 87 μg/ml) on 1 day in six replicates and on 3 separate days in triplicates. Robustness of the method was determined by employing two different sample weights of the aqueous extract (20 mg and 23 mg). Two different analysts carried out the analysis of the aqueous extract in three consecutive days to assess the ruggedness of the method. The sample solution was kept at 4°C and its stability was tested at 0, 7 and 44 h. Limit of quantification (LQ) and limit of detection (LD) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) following the International Conference on Harmonization Guidelines;[12] LQ = 10 (σ/S), LD = 3.3 (σ/S).

Statistical analysis

Data were expressed as mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance, unless otherwise indicated. If the overall *F* value was found statistically significant ($P < 0.05$), further comparisons among groups were made according to *post-hoc* Tuckey's test. All statistical analyses were performed using Statistical Package for the Social Sciences GraphPad In Stat 3 software (La Jolla, CA, USA).

RESULTS

HPLC standardization of the active extracts and fractions

Comparing the RP-HPLC chromatograms of *C. droserifolia*, it was evident that the aqueous extract contained the highest percent of the total active flavonol glycosides (78.20%, major peaks at R_t 7-10 min), followed by its ethyl acetate fraction (65.4%) (Figures 1 and 2). While, the 70% and 50% ethanolic extracts contained 51.17 and 42.66% of total flavonol glycosides, respectively (Figure 2).

The aqueous extract was standardized to contain not less than 1.5 ± 0.06% of F3 (kaempferol-4'-methoxy-3,7-dirhamnoside), while the ethyl acetate fraction was standardized to 2.7 ± 0.06% of F3.

In vivo antihyperglycemic activity

Both the aqueous and 70% ethanolic extracts of the aerial parts of *C. droserifolia* were chosen for the *in vivo* activity as they contained relatively high content of total active flavonol glycosides. They showed a significant antihyperglycemic response as they reduced the blood

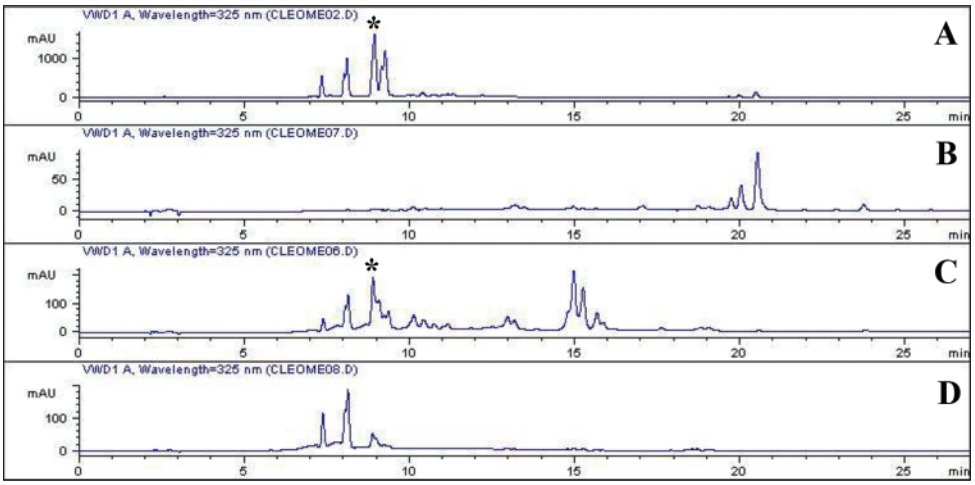

Figure 1: Reversed phase-high performance liquid chromatography chromatograms of the aqueous extract of *Cleome droserifolia* and its successive fractions. (a) Aqueous extract 50 g/L; (b) chloroform fraction 1 mg/ml; (c) ethyl acetate fraction 1 mg/ml; (d) butanol fraction 1 mg/ml; *compound F3 at R_t at 9.09.

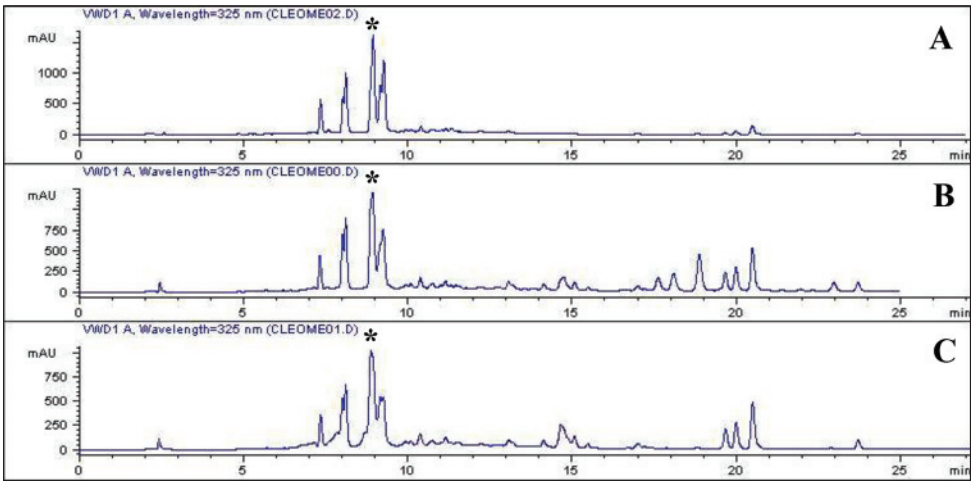

Figure 2: Reversed phase-high performance liquid chromatography chromatograms of the different extracts (50 g/L) of *Cleome droserifolia*. (a) Aqueous extract; (b) 50% ethanol extract; (c) 70% ethanol extract; *compound F3 at Rt at 9.09.

glucose levels by 47.6 and 38.5%, respectively, after 4 weeks of administration (Table 1). The ethyl acetate among other fractions of the parent aqueous extract showed the highest activity, as it caused 44.7% reduction in blood glucose level after 4 weeks of administration (Table 1).

The most active standardized aqueous extract was then tested at different dose levels (Table 2), the results showed that after 4 weeks the blood glucose levels were reduced by 31.25, 36.70 and 46.47% at a dose of 50, 75 and 100 mg/kg body weight, respectively.

Estimation of plasma insulin level

The aqueous extract moreover raised the levels of the blood insulin by 30.23, 92.78 and 146.24% at a dose of 50, 75 and 100 mg/kg body weight, respectively (Table 3).

Validation of the RP-HPLC method

Linear regression analysis of compound F3 was performed by plotting the mean peak area versus concentration (Figure 3). The correlation coefficient (R^2) of the standard calibration curve was 0.998 indicating linearity of the peak area in the range of 44-174 µg/ml. The LD and LQ for F3 were 0.01 and 0.02 µg/ml, respectively. Precision of the method during the intra- and inter-day run are given in Table 4. The relative standard deviation (RSD) was taken as a measure of precision. The data were within the acceptance criteria (< 5%). Sample of F3 proved to be stable in methanol solution within 44 h at 4°C (RSD 2.20%, Table 4).

Recovery tests were carried out to further investigate the accuracy of the method. The percent recovery of spiked

Table 1: Antihyperglycemic effect of the different extracts of *C. droserifolia*

Group	Time				
	0	2 weeks		4 weeks	
	M±SE (mg/dl)	M±SE	% of change	M±SE	% of change
Diabetic rats (Db) non treated	243.7±8.2	256.8±9.6	-	256.8±9.6	-
Db+metformin	257.3±11.4	129.8±4.3*	49.5	81.9±3.2*	68.2
Db+70% ethanolic extract	249.2±8.2	216.2±7.6*	13.2	153.2±4.6*	38.5
Db+aqueous extract	256.8±10.1	173.2±6.2*	32.5	141.9±5.5*	47.6
Db+*n*-hexane fraction	246.9±7.8	214.3±8.6*	13.2	198.6±7.1*	19.5
Db+chloroform fraction	251.9±8.6	186.8±7.4*	25.8	138.9±5.8*	41.8
Db+ethyl acetate fraction	258.4±7.1	187.4±6.3*	27.5	135.3±4.1*	44.7
Db+*n*-butanol fraction	258.3±10.2	224.9±8.4	13.3	203.7±6.5*	21.1

Extracts, fractions, and the standard metformin were given at a dose of 150 mg/kg body weight. *Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

Table 2: Antihyperglycemic effect of the different doses of the aqueous extract of *C. droserifolia*

	Blood glucose level mg/dl (M±SE)				
	Diabetic non-treated (Db)	Db+ 50 mg/kg	Db+ 75 mg/kg	Db+ 100 mg/kg	Metformin (150 mg/kg)
0	254.9±6.4	248.2±9.6	261.3±7.1	264.9±7.6	259.7±8.1
2 weeks	262.3±7.9	224.9±5.8	196.4±6.5*	192.3±6.2*	146.3±5.2*
4 weeks	258.9±7.7	178.4±4.9*	163.9±5.7*	138.6±4.3*	87.8±2.4*

*Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

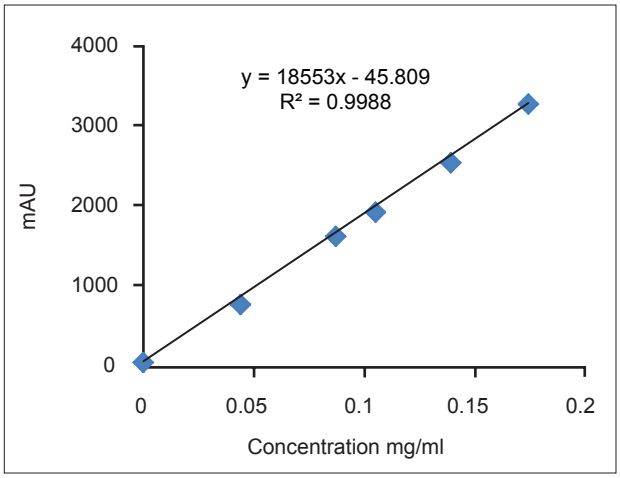

Figure 3: Standard calibration curve of the flavonoid glycoside F3 using reversed phase-high performance liquid chromatography.

aqueous extract samples with F3 sample at a concentration of 139 μg/ml was 100.5% with RSD of 2.25 (Table 5).

Influence of small changes in the chromatographic method, such as change in the sample weight and analyst, was also assessed to determine the robustness and ruggedness of the method, respectively. Results (RSD < 3%, Table 6) were also in favor of the developed RP-HPLC method.

DISCUSSION

In our previous work, the aqueous extract of the aerial parts of *C. droserifolia* was shown to have a similar effect to insulin in increasing the basal glucose up-take in cultured C2C12 skeletal muscle cells, while the ethanolic extract did not show any effect *in vitro*.[9] However, several studies proved the *in vivo* antihyperglycemic properties of the ethanolic extract at dose levels of 310 and 900 mg/kg

Table 3: Effects of oral administration of the different doses of the aqueous extract on plasma insulin levels in diabetic rats

	Blood insulin level uUL/ml (M±SE)			
	Diabetic non-treated (Db)	Db+ 50 mg/kg	Db+ 75 mg/kg	Db+ 100 mg/kg
0	8.9±0.1	8.6±0.1	9.7±0.4	9.3±0.2
2 weeks	8.1±0.1	9.4±0.2	14.6±0.5*	16.2±0.6*
4 weeks	8.5±0.1	11.2±0.3*	18.7±0.4*	22.9±0.7*

*Statistically significant difference from 0 time at $P<0.01$. M: Mean, SE: Standard error ($n=6$)

Table 4: Precision and stability of compound F3 ($n=3$)

Compound F3 (μg/ml)	Intra-day RSD (%)	Inter-day RSD (%)	Stability
44	4.25	3.82	
87	2.04	2.22	2.20

RSD: Relative standard deviation

Table 5: Recovery of compound F3 in the active aqueous extract ($n=3$)

Compound F3 original (μg/ml)	Spiked (μg/ml)	Found (μg/ml)	Recovery (%)	RSD (%)
139	199	199.99	100.50	2.25

RSD: Relative standard deviation

Table 6: Robustness and ruggedness of the RP-HPLC method (*n*=3)

		Mean (mg/ml)±SD*	RSD (%)
Ruggedness	Analysts (1 and 2)	0.064±0.0004	0.65
Robustness	20 mg ethyl acetate fraction	0.0628±0.0007	1.12
	23 mg ethyl acetate fraction	0.0631±0.0010	2.63

*Mean concentration of F3 in the aqueous sample as calculated from the standard calibration curve (*n*=3), RP-HPLC: Reversed phase-high performance liquid chromatography, SD: Standard deviation

body weight in alloxan-induced diabetic mice and rats, respectively.[2,3] By comparing the different extracting solvents using the RP-HPLC chromatographic profiles, it was evident that the aqueous extract was rich in the active flavonol glycosides followed by the 70% ethanolic extract, where the 50% ethanolic extract contained the least amount. Hence, both the aqueous and 70% ethanolic extracts of *C. droserifolia* were tested *in vivo* in alloxan-induced diabetic rats at 150 mg/kg body weight. The aqueous extract showed a higher activity. Consequently its four fractions were assessed for their activities. The ethyl acetate fraction, again containing relatively high content of the total flavonol glycosides, showed the highest activity, which was comparable to the mother aqueous extract. This strongly supports and confirms our previous *in vitro* assays, which showed that the ethyl acetate fraction and its isolated flavonol glycosides possessed significant insulin–like properties in peripheral tissues.[9]

The activity of the aqueous extract was thus tested at different dose levels, showing significant antihyperglycemic activities, especially at 100 mg/kg body weight, where its activity was 63.3% of that of metformin at 150 mg/kg body weight. Furthermore, oral administration of the aqueous extract for 4 weeks increased significantly the insulin blood levels, compared with the diabetic rats, which may be through stimulation of the activity of the remnant pancreatic β-cells.

This provided an explanation for the promising activity of the aqueous extract known traditionally strongly correlating it to the total flavonol glycosides content, and also suggested the potentiality of preparing a standardized aqueous extract of *C. droserifolia* for use against hyperglycemia.

Compound F3 was chosen as the bioactive marker for standardization of the aqueous extract being the major active compound which we previously isolated,[8,9] besides giving a sharp peak, which was not overlapping with other peaks using the developed fingerprint chromatogram.

To obtain a satisfactory chromatogram for the aqueous extract, several sample preparation methods were tried until reaching the optimum method, comparing the numbers, areas and resolution of the chromatographic peaks. The EXtrelut® prepacked column was conditioned with either 3 × 1 ml H_2O followed by 3 × 1 ml methanol or with methanol followed by water, when the aqueous extract (10 mg) was dissolved in 1 ml methanol or in H_2O, respectively. Elution was carried out with different mixtures of methanol: H_2O (25, 30, 40, 50, 75 and 100%).

In order to obtain valid chromatographic conditions, different RP-HPLC parameters were compared and optimized, including mobile phases (methanol/water and acetonitrile/water with different modifiers as acetic acid and *O*-phosphoric acid), various gradient and isocratic elutions, as well as, different flow rates. Best conditions were determined and the established aqueous extract chromatogram was used for standardization of the extract.

The active aqueous extract was standardized to contain not <1.5 ± 0.06% of F3 (kaempferol-4'-methoxy-3,7-dirhamnoside).

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Comparative Morphological and Anatomical Study of *Onosma caucasica* Levin. ex M. Pop. and *Onosma sericea* Willd. (Boraginaceae Juss.)

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ABSTRACT

Introduction: Finding sources of drugs among the species of genus *Onosma* of the local flora is actual direction. Many of the species of genus *Onosma* are weeds, some live exclusively on dry sandy or rocky slopes. Many of the species of genus *Onosma* have used in ethnopharmacology as anti-inflammatory, analgesic, and wound health remedies. The purpose of this research is morphological and anatomical study of herbs of *Onosma caucasica* Levin. ex M. Pop. and *Onosma sericea* Willd. (Boraginaceae). **Materials and Methods:** The objects of the study were collected in North Caucasus (Russia) in June 2011. Materials morphological study herbarium specimens were stored in the herbarium fund the Department of Botany (acronym PGFA). Materials micromorphological study were temporary slides are the leaf, stem and flowers. **Results:** The morphological results were compared with the Flora of the USSR. Anatomical characters of leaves and stems of the species were observed to be similar to the usual features of Boraginaceae anatomy. All results are supported by photographs. The nature of trichomes on the leaf blade has a major diagnostic value in determining the authenticity of herbs the studied species. **Conclusion:** The severity of downy leaf blade and the corolla, and the structure of the hair can reliably identify this type of raw material and serve as the basis for the development of the regulatory documentation for its standardization.

Keywords: Anatomy, Boraginaceae, morphology, *Onosma*

INTRODUCTION

According to the "Takhtajan system of plant classification" genus *Onosma* belongs to the family Boraginaceae Juss., subfamily boraginoideae, part of the tribe *Lithospermeae*.[1] The International Plant Names Index includes 363 species of this genus.[2] According to the "Flora of China" in Asia found 145 species, 29 of which in China.[3] On the territory of Russia and adjacent states (within the territory of the former USSR) genus is represented by 51 species: the flora of Eastern Europe (20 species), Caucasian (21 species), West Siberia (four species), East Siberia (three species), Central Asia (20 species).[4]

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On the territory of Russia registered two medicinal products containing as a component of the extract of the herb *Onosma bracteatum*:

- "Cystone" (Himalaya Drug Company, India) - reduces the irritation of the mucous membrane of the urinary bladder, anti-inflammatory, anti-microbial, antiseptic and diuretic effect.
- "Linkus" (Herbion International Inc., Pakistan) - reduces the intensity of coughing, has expectorant, mucolytic, and anti-inflammatory effects.

Finding sources of drugs among the species of genus *Onosma* of the local flora is actual direction. Many of the species of genus *Onosma* are weeds, some live exclusively on dry sandy or rocky slopes. Ecology of their habitat is different, but they share a permanent morphological marker, as the nature of hard-fibrous hairs formed characteristic hairs.[5-7] The representatives of the family is *Onosma caucasica* Levin. ex M. Pop., which grows in all regions of the Caucasus on the rocky slopes, cliffs and talus, and *Onosma sericea* Willd., which

grows on rocky slopes, in the lower and middle mountain zones in Dagestan, as well as in Central Asia. Chemically considered species are not well-investigated. There are found naphthoquinones (shikonin), flavonoids (astragalin, hyperoside, 3,7-diramnozidquercetin 7-glucoside, luteolin, kaempferol, quercetin) in raw *O. caucasica*.[8]

O. sericea contains naphthoquinones (derivatives of shikonin). It is used to treat wounds in folk medicine in Turkey. The ethanol extract of the experiment has anti-inflammatory and analgesic properties.[9,10]

Many of the species of genus *Onosma* have used in ethnopharmacology.[11] Purpose of this research is morphological and anatomical study of herbs of *O. caucasica* and *O. sericea*.

MATERIALS AND METHODS

The object of the present study were herbarium specimens *O. caucasica*, collected in June 2011 on the slopes of Mount Mashuk, samples of raw *O. caucasica* and *O. sericea*, collected in Dagestan in June 2011. Materials morphological study herbarium specimens were stored in the herbarium fund the Department of Botany (acronym PGFA). Materials micromorphological study were temporary slides are the leaf, stem and flowers. Sections were fixed in the glycerol-water solution staining was performed with sulfuric acid and phloroglucinol.

Dried raw material is a mixture of leafy stems with flowers and fruits of various degrees of development and individual stems, whole or crushed leaves, flowers, rare fruits. To identify the anatomical and diagnostic features the raw material was prepared by classical methods of pharmacognosy.[6,7,12] The study was performed using a microscope "Micromed-1" with trinocular head, with lenses ×4, ×10, ×40, eyepieces ×10. Photomicrography made with a digital camera Electronic Eyepiece MD300 (3.1 megapixels).

RESULTS

In carrying out morphological studies revealed as morphological characteristics.

O. caucasica Levin. ex M. Pop (Figure 1) is half-shrub up to 40 cm is characteristic structure of the root system, it is represented by the main root system in the upper part of the main root is formed branched caudex, covered with remnants of leaves, it is a combination of lower long-term the resumption of the shoot in ascending order herbaceous

Figure 1: *Onosma caucasica* Levin. ex M. Pop. General appearance, Pyatigorsk, mount. Mashuk in June 2011: (1) fragments of leaves and stems; (2) inflorescence; (3) leaves; (4) chopped raw material; (5) grass powder.

perennials. Escape elongated, upright. Phyllotaxy another. A sheet of plain, sedentary. Leaf blade one-piece, form widely-elliptical edge solid, base wedge-shaped, tip pointed, pressed bristly pubescence. Inflorescence terminal: Cimoids, thyrsus of monohazy. Inflorescence is a partial curl.

Actinomorphic flowers, calyx about 15 mm, gray-pressed-pubescent. Whisk sympetalous consists of five petals up to 30 mm in length, without bending. Petals are yellow. Androecium consists of five stamens. Gynoecium coenocarpic consists of two fused carpels. The fruit-dry nut, 3.5 mm in length with a short beak.[13-15]

O. sericea Willd. (Figure 2) is a perennial herb 20-40 cm tall. The whole plant is covered with appressed silvery hairs. Calyx in fruit growing. It grows on rocky slopes from the bottom to the middle belt of the Central Dagestan. Leaves sessile, silver-grayish, lower-elliptical-oblanceolate, the upper-lanceolate. Flowers in many-flowered curls. Corolla about 20 mm long, 1.5-2 times longer than the calyx, yellowish-white.[13-15]

In conducting the study micromorphological grass *O. caucasica* Levin. ex M.Pop. (Figure 3-1) and *O. sericea* Willd. (Figures 3-2) considered especially micromorphological structure of the stem, petiole and leaf blade in cross sections, as well as the structure of the epidermis and the leaf blade elements flowers on preparations from the surface.

The cross section of the stem of *O. caucasica* has a cylindrical shape (Figure 4). The structural composition-investing tissue, the cortex, the central cylinder. The cover fabric-shows the epidermis, consisting of a single layer of living cells, parenchymal shape, coated on the outside cuticle. The stem has a characteristic pubescence formed following types of trichomes: Sit-branched hairs, simple, unicellular, capitate hairs and glandular hairs. The structure of the hair cells of uniform, covered with warty cuticle. Bark is located under the epidermis is composed of loose collenchyma and performing parenchyma. Endoderm is weak. Collenchyma composed of living cells, rounded, with irregularly thickened cell walls. It is one, at least two layers of the epidermis below.

Figure 2: *Onosma sericea* Willd. General appearance, in Dagestan in 2011: (1) Fragments of leaves and stems; (2) inflorescence; (3) leaves; (4) chopped raw material; (5) grass powder.

Chlorenchyma is a lively, rounded or polygonal cells containing chloroplasts. Performs live with thin-walled parenchyma presented fairly large cells. Axial cylinder consists of a pericyclic zone, phloem, cambium and xylem parenchyma and core. Pericyclic zone consists of a single layer of cells, pre-dominantly parenchymal, then there are also elements of the sclerenchyma.

Non-beam characteristic type of structure of the conducting system. Phloem consists of fine sieve elements and parenchymal cells. Xylem is separated from the phloem and cambium is a fairly large vessels located close to the core of parenchyma and small tracheids, concentrated closer to the cambium. Tracheids are rectangular in cross section and strongly lignified wall. Type staley-siphonostele. In the central part of the core located parenchyma. The core is composed of a parenchymal cell. In the central part of the core breaks the parenchyma, forming cavity. The epidermis of the stem is represented by living cells slightly elongated shape with beveled ends. Stomatal apparatuses anomocytic type are rare. Trichomes in the form of a simple single-celled hairs closer to the veins. Cuticle hair is warty cuticle.

The structural composition-investing tissue, mesophyll conductive beam, mechanical tissue. Type of coating fabrics-the epidermis, with a lot of simple multicellular

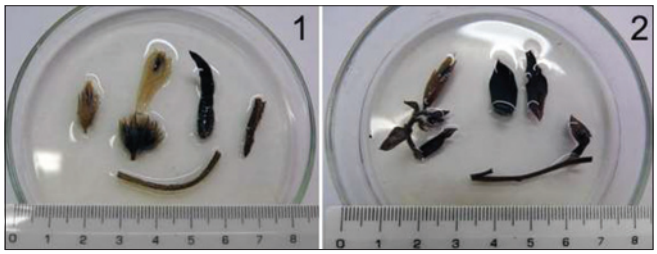

Figure 3: Comparative characteristic morphological signs of *Onosma caucasica* Levin. ex M.Pop. (1) and *O. sericea* Willd. (2).

Figure 4: *Onosma caucasica* Levin. ex M. Pop.: (1 and 2) Cross-section of stem. (painting floroglucin and concentrated hydrochloric acid): (a) epidermis, (b) trichomes, (c) collenchyma, (d) phloem, (e) cambium, (f) xylem, (g) parenchyma; (3 and 4)-cross-section of leaf: (a) Upper epidermis, (b) palisade parenchyma, (c) spongy parenchyma, (d) vascular bundle, (e) lower epidermis; (5) glandular hairs; (6) structure of trihomes; (7 and 8) stellate hairs; (9) upper epidermis; (10) lower epidermis; (11) simple conical-celled hairs; (12) corolla; (13) calix; (14) pollen (scale value 10 μm).

hairs, a single layer of living parenchyma cells outside covered with cuticle. Mesophyll can be differentiated into palisade and spongy. Palisade mesophyll is only below the upper two layers of the epidermis. Cells are flush with each other, contain chloroplasts. Spongy mesophyll presented assimilation spongy parenchyma cells are living, thin-walled, round, contain chloroplasts. In the main vein of the mesophyll cells have collenchymal thickening. Conductive beam 2, they closed the collateral, are located in the central vein. Conductive bundle consists of phloem and xylem. Xylem is oriented towards the upper side and the lower side phloem lamina. The conductive beam is accompanied by mechanical tissue - sclerenchyma located around vascular bundle.

The upper epidermis of the leaf blade is composed of the basic cells of the epidermis, stomata and trichomes. Anticlinal cell walls of the main straight or slightly wavy, the shape of cells-isodiametric. Stomatal apparatuses anisocytical and anomocytical type. Compared with the lower surface lamina stomatal apparatus present in minor amounts. Trichomes are either simple single-celled hairs

with warty cuticle, or assembled into conglomerates as sedentary multicellular hairs with warty cuticle.

The bottom epidermis of the leaf blade is different top lots of stomatal apparatuses. Anticlinal walls of epidermal cells remain straight shape. Stomatal apparatuses anomocytic and anisocytic type. Trichomes are on the lower side lamina. The epidermis is a corolla basic cells and trichomes. Anticlinal cell walls of the main straight. The rectangular shape of the cells, the cells with elongated rounded ends. Trichomes are fairly common, represented by simple unicellular outgrowths. In reviewing the slides sheet from the surface of *O. sericea* found that trichomes and stomata are on both sides of the leaf blade. The cells of the upper and lower epidermis shapes with straight walls. Stomatal apparatus anomocytic type.

Numerous trichomes are two types of hair: (1) Large emergent like simple unicellular hairs with rough-warty surface, surrounded by a rosette of epidermal cells, which

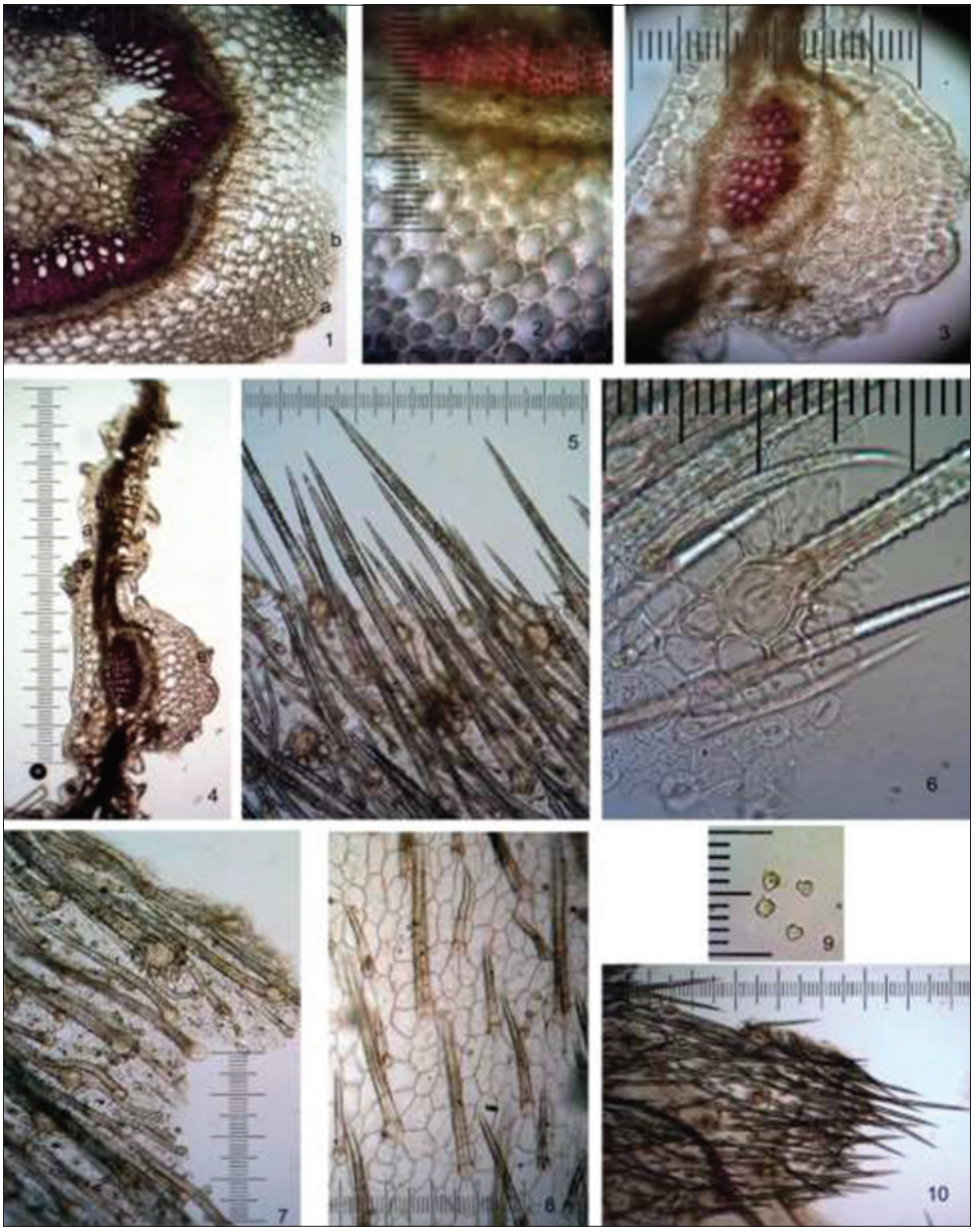

Figure 5: *Onosma sericea* Willd.: (1 and 2) Cross-section of stem (painting floroglucin and concentrated hydrochloric acid): (a) Epidermis, (b) collenchyma, (c) phloem, (d) cambium, (e) xylem, (f) parenchyma; (3 and 4) cross-section of leaf: (a) Upper epidermis, (b) palisade parenchyma, (c) spongy parenchyma, (d) vascular bundle, (e) lower epidermis; (5) emergent like simple unicellular hairs; (6) upper epidermis; (7) lower epidermis; (8) corolla with simple conical-celled hairs; (9) pollen; (10) calix (Scale value 10 μm).

are often present colored contents, and (2) simple conical-celled hairs. The hairs are bent and the ends are directed towards the top of the sheet.

The cross section of the stem of *O. sericea* has a round shape (Figure 5). The surface has a sparse pubescence. Hairs simple, one-celled with warty cuticle. The cover fabric is represented by the epidermis, followed by collenchyma plate-type. Collenchyma cells are deposited in the form of continuous layers 4-7. Cortical parenchyma represented by large thin-walled cells isodiametric.

The vascular system of the stem is non-beam type of structure. By type Staley this type of stem can be attributed to siphonostele. Phloem occupies a small volume. Xylem vessels is presented, tracheids and lignifies parenchyma (painting floroglucin and concentrated hydrochloric acid).

Table 1: Comparative anatomical measurements of *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop.

	O. caucasica		*O. sericea*	
	Length (µm)	Breadth (µm)	Length (µm)	Breadth (µm)
Leaf				
Upper epidermis	40-50	30-40	30-50	30-40
Lower epidermis	30-50	30-40	30-40	20-30
Simple conical-celled hairs	200-400	20-30	160-180	10-15
Emergent like simple unicellular hairs	800-1700	50-60	480-860	20-40
Stomata	30-40	20-25	25-30	20-25
Stem				
Epidermis cells	30-40	30-40	20-40	20-40
Collenchyma cells	30-50	30-50	20-40	30-50
Diameter of trachea	-	10-30	-	10-20
Diameter of vessel	-	40-60	-	20-70
Diameter of pith cells	-	70-120	-	30-70
Flower				
Epidermis	60-130	30-60	70-140	30-70
Pollen	15-20	15-20	15-20	15-20
Trichomes				
Simple conical-celled hairs	70-270	20-30	170-460	20-25
Glandular hairs	20-40	10-15	-	-

O. caucasica: Onosma caucasica, O. sericea: Onosma sericea

Radial rows of receptacles arranged closer to the core are more numerous. The core is composed of a parenchymal cell. In the middle of the stem has a cavity. The epidermis is a corolla of *O. sericea* thin-walled cells rounded. On the surface, there are numerous simple unicellular hairs with thick warty surface.

Measurement of anatomic signs is carried out (Table 1).

DISCUSSION

Morphological, micromorphological and anatomical characteristics of *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop. were examined in this study. There are a few information about morphological properties of *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop. in the Flora of USSR.[13] Results of comparison of morphological properties both genus are presented in Table 2.

The anatomical properties *O. sericea* Willd. *O. caucasica* Levin. ex M. Pop. have the general characteristics of family Boraginaceae, as *O. caucasica* Levin. ex M. Pop. refers to sub *Asterotricha* Boiss. group Oxianna M. Pop., which differs from the presence of large steel bristles with stellate hairs. *O. sericea* Willd. refers to the section Aponosma DC., which has a more long setae on simple star-shaped tubercles not.[13] Results of comparison of anatomical properties both genus are presented in Table 3.

CONCLUSION

The study identified the main diagnostic features of herb *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop.

Our analysis shows that, in determining the authenticity of herb *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop. hairs on the nature of the leaf blade has a major diagnostic value. The severity of downy leaf blade and the corolla,

Table 2: Comparative morphological properties of *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop.

	O. caucasica	*O. sericea*
Distribution	Caucasus (all areas) On stony slopes, Screes, cliffs	Central Asia, Caucasus, Eastern and Southern Caucasus On rocky slopes, on average mountain belt of oak forests
Life form, stems	half-shrub, 20-40 cm in height, branching stems and simple, densely pubescent tough fibrous	Half-shrub, 30-50 cm in height, branching stems, densely pubescent, snuggled, white and silky
Foliage	Form of the leaf blade-narrowly lanceolate, gray-green, pressed from the bristles on the edges wrapped, 10 mm wide	Form of the leaf blade-elliptical, the lower-wider, elliptical-oblanceolate, the upper - lanceolate, sessile, silver-grayish
Inflorescences	Monohazy curl polyanthous	Paniculata thyrsus of 2-3 curls
Flower	A cup with a little fruit growing, from 8 to 18 mm Pubescence cup white appressed bristly Corolla tubular, palely yellow, then reddish	Calyx with short tube, with fruits angular, strongly projecting through the veins Pubescence cup firmly pressed, white and silky Whisk in 2 times longer than the calyx, whitish-yellow color

O. caucasica: Onosma caucasica, O. sericea: Onosma sericea

Table 3: Comparative anatomical properties of *O. sericea* Willd. and *O. caucasica* Levin. ex M. Pop.

	O. caucasica	*O. sericea*
Leaves Cross-section	Dorsoventral Collenchymas 2-3 layers mesophyll cells have collenchymal thickening. Conductive beam 2, they closed the collateral, are located in the central vein	Dorsoventral Collenchymas 2-3 layers mesophyll cells have collenchymal thickening. Conductive beam 2, they closed the collateral, are located in the central vein
Lower epidermis	Basic cells of the epidermis (straight or slightly wavy), stomata and trichomes	Basic cells of the epidermis (straight or slightly wavy), stomata and trichomes
Upper epidermis	Basic cells of the epidermis (straight or slightly wavy), stomata and trichomes	Basic cells of the epidermis (straight or slightly wavy), stomata and trichomes
Stomatal type	Anomocytic and anisocytic	Anomocytic and anisocytic
Trichomes type	Stellate hairs; Simple conical-celled hairs; Glandular hairs	Emergents like simple unicellular hairs; Simple conical-celled hairs
Stem structure	Cylindrical shape Non-beam type	Cylindrical shape Non-beam type
Calyx structure	A lot of simple conical-celled hairs	
Corolla structure	A few of simple conical-celled hairs	A lot of simple conical-celled hairs

O. caucasica: Onosma caucasica, O. sericea: Onosma sericea

and the structure of the hair can reliably identify this type of raw material and serve as the basis for the development of the regulatory documentation for its standardization.

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Pharmacognostic and Phytochemical Studies on Flowers of *Aerva lanata* [L.] Juss. ex. Schult

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ABSTRACT

Background: *Aerva lanata* is an interesting plant used in traditional medicine for many years and used for the treatment of bladder and kidney stones. **Objective:** The aim was to study detailed pharmacognostic profile of an important medicinal plant in the Indian system of medicine, *A. lanata* (amaranthaceae). **Materials and Methods:** Flower samples of *A. lanata* were studied by macroscopical, microscopical characters. Physicochemical, phytochemical, and fluorescence analysis of powder of the plant was performed according to the methods of standardization recommended by World Health Organization. **Results:** Macroscopically flowers are small, actinomorphic, and solitary or aggregated in cymes. Microscopically ovary was found to be bicarpellary, syncarpous, unilocular, superior; ovules one to many and campylotropous type. Powder microscopy of flower revealed the presence of epidermis with stomata and covering trichomes, calcium oxalate crystals, starch grains, and oil globules. The investigations also included fluorescence analysis. Physiochemical parameters such as total ash, acid insoluble ash sulfated ash and water soluble ash; moisture content values were found to be 12.66%, 1.64%, 9.12%, 4.52%, 12%, respectively. Preliminary phytochemical screening showed the presence of carbohydrates, triterpenoids, flavonoids, glycosides, and phenolic compounds. **Conclusion:** The results of the present study can serve as a valuable source of information and provide suitable standards for identification of this plant material in future investigations and applications.

Keywords: *Aerva lanata*, fluorescence, microscopic, physiochemical, phytochemical

INTRODUCTION

Medicinal plants are used by nearly all cultures to prevent or treat illness. Many of most common medicines of today are developed from the components of medicinal plants. *Aerva lanata* (amaranthaceae) is an interesting plant used in traditional medicine for many years and described as one of the best known remedies for the treatment of bladder and kidney stones. It is a common weed grows wild everywhere in the plains of India, commonly known as "Mountain knot grass."[1] It has been ethno medicinally used as a therapeutic agent for a variety of diseases.[2-5] Flowers are used in dysentery, diarrhea and bronchitis.[6] However, it is important to conduct thorough investigation of many traditionally used medicinal plants with reference to modern system of medicine.[7] A regular and wide spread use of herbs throughout the world has increased serious concerns over their quality, safety and efficacy.[8] Hence, pharmacognostical study gives the scientific information regarding the purity and quality of the plant drugs.[9] The objective of the present study is to evaluate various pharmacognostical parameters such as macroscopic, microscopic, physicochemical, fluorescence, and phytochemical studies of the plant.

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MATERIALS AND METHODS

Plant material

A. lanata plants were collected from Bhimavaram, East Godavari District, Andhra Pradesh. Botanical identification of the plants was done by Dr. P. Bangara Raju, Department of Botany, DNR College, Bhimavaram. Specimens of *A. lanata* (Voucher number: SVCP/Cognosy/1) was conserved in Shri Vishnu College of Pharmacy, Bhimavaram.

Pharmacognostic study

Macroscopy

Fresh flowers were taken for morphological and histological studies. Coarse powder (60#) was used to study microscopical characters, physicochemical parameters and for phytochemical investigation. Microscopical studies were performed as per standard procedure.[10-12] The powder microscopy was performed according to the method of Khandelwal.[11]

Extraction of plant material

The flowers were dried under shade for 15 days. The material was pulverized and powder was extracted with methanol. The extract was evaporated to dryness under reduced pressure at 45°C to give a solid residue. The residue was weighed and stored in refrigerator for further phytochemical study.[13]

Physicochemical and phytochemical analysis

Physicochemical values such as ash values and extractive values were determined according to the well-established official method and procedure.[14-17] Preliminary screening was carried out using the standard procedure described by Khandelwal.[12,16]

Florescence analysis

Powdered leaf material was treated with various chemical reagents and exposed to visible, ultraviolet (UV) light (short and long UV) to study their fluorescence behaviour.[18,19]

RESULT AND DISCUSSION

Taxonomic classification

Kingdom: Plantae, Subkingdom: Viridaeplantae, Phylum: Tracheophyta, Subphylum: Euphyllophytina, Class: Spermatopsida, Subclass: Caryophyllidae, Superorder: Caryophyllanae, Order: Caryophyllales, Suborder: Chenopodiineae, Family: Amaranthaceae, Subfamily: Amaranthoideae, Tribe: Amarantheae, Genus: *Aerva,* Specific epithet: *lanata,* Botanical name: *A. lanata* (L) Juss.ex Schult.

Synonyms: *Achyranthes lanata* L., *Achyranthes villosa* Forssk, *Aerva sansibarica* Suess, *Aerva incana* Suess.[20,21]

Common names

Bengali: Chaya. Hindi: Gorakhbuti or Kapuri jadi, Kannada: Bilesuli, Malayalam: Cherula, Marathi: Kapuri-madhura, Punjabi: Bui-kaltan (flowers as sold in bazaars), Rajasthani: Bhui, Sanskrit: Astmabayda, Sindhi: Bhui, Jari, Tamil: Sirru -pulay-vayr, Telugu: Pindi-kura, Pindi-chettu.[22]

Macroscopic characteristics

Flowers are greenish white, very small. The tiny clusters of two or three flowers grow in the leaf axils, 1.4-1.5 cm long and 3-4 mm wide. Flowers spikes are stalk less, solitary or usually in clusters, divergent, cylindrical, silky white to creamy, forming a long inflorescence leafy to the ultimate spikes. Flowers are about 0.1 inches (2.5 mm) long, sessile, often bisexual, in small dense sub sessile axillary heads or spikes 6-13 mm long, often closely crowded and forming globose clusters; bracteoles 1.25 mm, long, membranous, broadly ovate, concave, apiculate. Tepals 3-5, stamens as many as tepals and opposite to perianth members, rarely fewer than tepals; filaments free, connate into a cup at base or entirely into a tube, pseudostaminodes present or absent; anthers uni or bilocular, dorsifixed, introrsely dehiscent. Ovary bicarpellary, syncarpous, unilocular, superior; ovules one to many campylotropous; style persistent, short and indistinct or long and slender; stigma capitate, penicillate, bilobed or forming two filiform branches. Perianth 1.5-1.25 mm long; sepals oblong, obtuse, sometimes apiculate, silky-hairy on the back.[1,22-25] Utricle broadly ovoid, acute; stigmas two (Figure 1). Inflorescences are elongated or condensed spikes (heads), racemes, or thyrsoid structures of varying complexity. The dried flowers which look like soft spikes are sold under the commercial names as buikallan or boor. It is one of the plants included in dasapushpam, the 10 sacred flowers of Kerala. The flowers are normally self-pollinated. Flowering time is from May to October.

Microscopy

The powder microscopy of flower shows trichomes, pollen grains, starch grains, calcium oxalate crystals,

Figure 1: Morphology of *Aerva lanata* (a) plant (b) flowers.

epidermal cells and stomata. Trichomes are multicellular, uniseriate with spinulated surface, tapered at the end and multiarticulate. Pollen grains are spherical in shape and are about 17-20 μm in diameter. Starch grains are oval to ellipsoidal, mostly simple, without any striations. Calcium oxalate crystals are rosette shaped. Epidermal cells are with almost straight walls and contain anomocytic stomata (Figure 2).

Physicochemical and phytochemical analysis

The yield of the methanolic extract was 2.1 g/Kg. Phytochemical studies revealed the presence of carbohydrates, triterpenoids, flavonoids, glycosides and phenolic compounds. The total ash, acid insoluble ash sulfated ash and water soluble ash; moisture content values were found to be 12.66%, 1.64%, 9.12%, 4.52%, 12% respectively. Results of florescence analysis were summarized in Table 1.

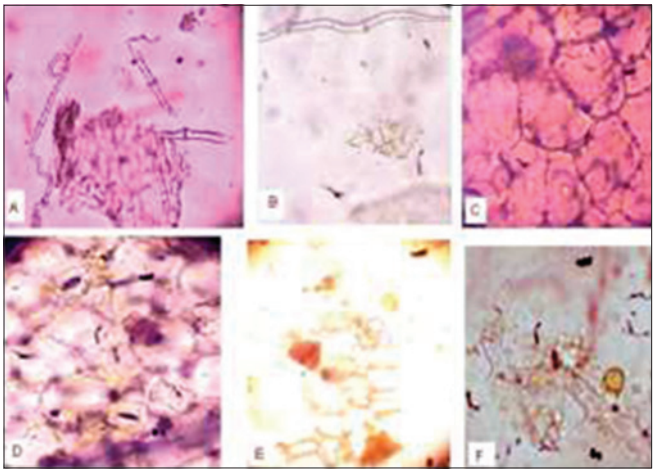

Figure 2: Powder microscopy of flowers (a) trichome (b) trichome and epidermis (c) epidermal cell (d) stomata (e) starch grains (f) pollen grain.

Table 1: Fluorescence characteristics of *Aerva lanata* flower powder

Reagent	Normal light	Under UV light	
		254 nm	365 nm
Dry powder	Whitish green	Whitish green	Whitish green
Powder+Dil./Conc. HCl	Whitish green	Brownish green	Brownish green
Powder+Dil. H_2SO_4	Whitish green	Green	Green
Powder+Conc. H_2SO_4	Brownish green	Blue fluorescence	Blue fluorescence
Powder+Dil. Ammonia	Pale green	Blue fluorescence	
Powder+$FeCl_3$	Yellow	Blue fluorescence	Blue fluorescence
Powder+Iodine solution	Brown	Brown	Blue fluorescence
Powder+Bromine water	Pale brown	Brown	Blue fluorescence

UV: Ultraviolet, Dil.: Dilute, Conc.: Concentrated

CONCLUSION

It is concluded that the above pharmacognostic and phytochemical parameters are very useful for the identification and authentication of the species. The results of the present study will also be helpful in preparation of monograph. The reported phytochemical and pharmacological studies on the species support and proved its traditional uses. Further research will help in the isolation of active compounds for therapeutic importance.

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Pharmacognostic Investigation of *Valeriana hardwickii* Wall. A Threatened Herb

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ABSTRACT

Aim: *Valeriana hardwickii* Wall. belongs to family Valerianaceae grown in high altitude areas of north west Himalaya to Bhutan. Traditionally, the roots of the plant are used to treat insomnia; however, no reports are available regarding any pharmacognostic work on this plant. **Material and Methods:** The study includes morphological, microscopic and preliminary phytochemical investigations of the roots and rhizome. Anatomical studies of roots and rhizome shows the presence of diagnostic characters such as thick walled cortex cells, annular xylem fiber, pitted xylem vessel and rhizome hair. **Results:** The preliminary phytochemical screening of petroleum ether, dichlomethane and aqueous extract revealed the presence of tritepenoids, alkaloids, irridoidal glycosides and flavanoids. The study was carried out as per WHO guidelines. **Conclusion**: The outcome of this work will help the researchers to differentiate *V. hardwickii* from the other species of valerian.

Keywords: Pharmacognostic investigation, phytochemical screening, valerianaceae, *Valeriana hardwickii* Wall

INTRODUCTION

Valeriana hardwickii Wall. (Family: Valerianaceae) is a threatened medicinal herb distributed in high altitude areas of Himalayas.[1] The roots and rhizomes are popularly called as valerian and useful as sedative,[2] diuretic,[3] anthelmentic[4] and antidiarrheal.[5] α-epikessyl glycol diacetate, kessyl acetate,[6] borneol acetate,[7] and epoxysessquithujene[8] were reported from essential oil.

V. hardwickii is under threat due to its over exploitation in their natural habitat. The roots and rhizome are substituted for *Valeriana wallichi* commercially. There is a scarcity of pharmacognostic and pharmacological data on *V. hardwickii*. In the present study, we have reported macroscopic, microscopic, powder analysis in different solvent, which would help in proper authentication of dried plant and powdered drug.

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MATERIALS AND METHODS

The roots and rhizomes of *V. hardwickii* were procured from procured from Natural Remedies Pvt., Ltd., Bangalore. Authentication of the plant sample was done by Dr. B.D Huddar, Head, Department of Botany, HSK College of Science and Arts Hubli. Voucher specimen (NRVP-03) was deposited in Department of Pharmacognosy and Phytochemistry, KLES College of Pharmacy, Hubli. The phytochemical parameters such as loss on drying,[9] extractive values were carried out as per official standard procedure.[10] Anatomical investigation was done by microtome sections and powder microscopy was performed according to the prescribed procedure.[11,12] The dried roots of *V. hardwickii* were pulverized. 100 g of coarse powder was successively extracted with petroleum ether (40-60°C) and dichloromethane at 40°C in a soxhlet extractor. The marc left after extraction was refluxed thrice with water on a water bath for 4 h. The extract was concentrated in a rotary flash evaporator and residue was dried in a desiccator.

The extracts were subjected to qualitative chemical examination to detect the presence of various phytoconstituents.[13,14]

RESULTS AND DISCUSSION

The systematic pharmacognostical study will give valuable information for the future studies.

Macroscopy

Macroscopiclly the roots and rhizomes are brownish in color. Rhizomes are short; roots are slender, 1 mm in diam. Stolons one to several, or absent. Stems are erect, often hispidulous below, glabrous above except at nodes. Pieces of rhizome are often contain longitudinal striations. Strong, unpleasant odor and bitter taste.

Microscopy

Transverse section of roots and rhizome

Transverse section of *V. hardwickii* shows outermost cortex consist of rectangular to squarish thick walled cells; pericyclic fiber containing 8-10 layers of isodiametric prarenchymatus cells; cambium region contain alternate arrangement of phloem and xylem. Meta xylem and protoxylems were present in cambium. Central pith region contain parenchymatous cells (Figure 1a).

Powder characteristics

A grayish-brown powder with a strong odor and a bitter taste. The abundant thick walled paranchymatus cells of the cortex filled with starch grains. Cells are fairly large, round, moderately thickened walls. Annular shaped lignified xylem fibers. The phloem fibers are long, thin walled, non-lignified with blunt ends. The pitted xylem vessels occur in small groups, lignified, usually reticulate thickened, small bordered pits at the base. A special character rhizome hair with unicellular, pointed end with circular base was observed (Figures 1b-f).

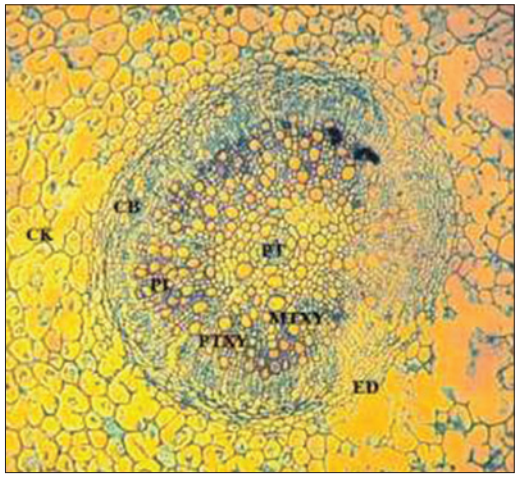

Figure 1a: Transverse section of rhizome. CK-Cork cells; ED-Endodermis; CB-Cambium; PL-Phloem fibre; PTXY-Protoxylem; MEXY-Meta xylem; PT-Pith region

Physicochemical characters

The results of the physical constants of the drug powder are given in Table 1. Total ash of crude powder of *V. hardwickii* Wall. was 5.03 ± 0.19% and sulfated ash was 12.09 ± 0.39%. Low total ash and sulfated ash signifies that less inorganic matters are present in drug. Extractive yield was highest in water (28.12 ± 0.54%), followed by alcohol (21.26 ± 0.18 %) and lowest yield was in petroleum ether (0.591 ± 0.06%). The results suggest high polar constituents are present in drug.

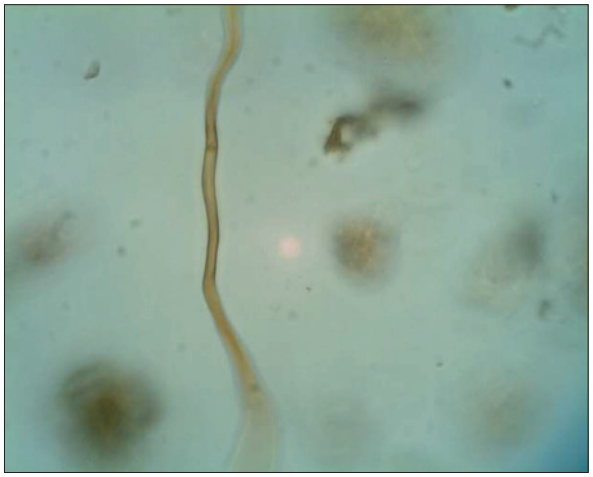

Figure 1b: Phloem fiber.

Table 1: Physicochemical characterization of powder of *Valeriana hardwickii* Wall

Parameters	Estimated value %w/w
Ether soluble extractive value	0.591±0.06
Alcohol soluble extractive value	21.26±0.18
Water soluble extractive value	28.12±0.54
Moisture content	8.23±0.11
Total ash	5.03±0.19
Sulfated ash	12.09±0.39

Table 2: Preliminary phytochemical screening of *Valeriana hardwickii* Wall

Type of phytoconstituent	Petroleum ether extract	Dichloromethane extract	Aqueous extract
Alkaloids	-	+	+
Steroids	+	-	-
Carbohydrates	-	-	+
Phenolic (Flavonoids)	-	-	+
Proteins and amino acid	-	-	-
Glycoside (Iridoids)	+	+	-
Triterpenoid	+	-	-
Tannins	-	-	+

+: Present, -: Absent

Figure 1c: Root hair.

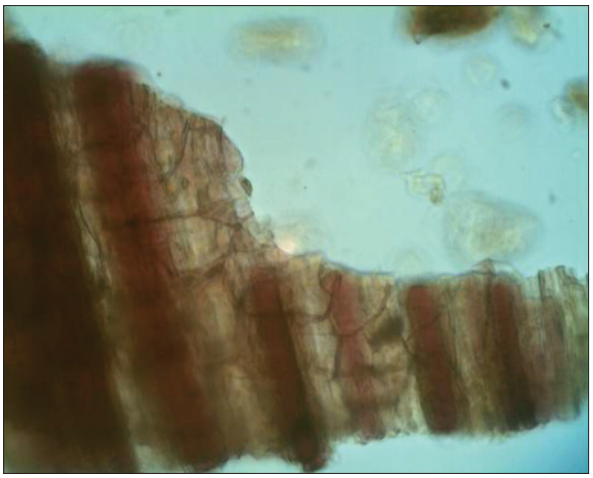

Figure 1e: Xylem bands.

Figure 1d: Thick walled cortex.

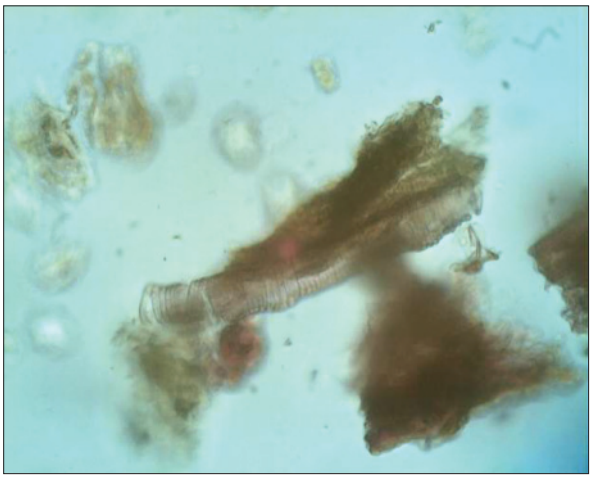

Figure 1f: Pitted xylem vessel.

Phytochemical screening

Preliminary phytochemical screening is tabulated in Table 2. The results point out the presence of steroids, triterpenoids and iridoidal glycosides in petroleum ether extract. Alkaloids and iridoids in dichloromethane extract and aqueous extract contain alkaloids, flavonoids, and tannins.

CONCLUSION

The pharmacognostical study is one of the important tools in the identification of crude drugs. The present study provides useful information and helps in the correct identification of roots and rhizome of *V. hardwickii* Wall. In addition, the results of the present study could be useful for preparation of a monograph of the plant.

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Evaluation of Wound Healing Potential of Some Indian Herbal Extracts and it's Formulation in Acne Vulgaris

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ABSTRACT

Objective: *Butea monosperma, Barleria prionitis, Casuarina equisetifolia, Dalbergia sissoo,* and *Lagenaria siceraria* are reported to possess anti-acne, anti-inflammatory, anti-ulcer, and wound healing activity. The present work is aimed at evaluating the wound healing potential of these plant materials in acne vulgaris. **Methodology:** Individual gel formulations containing extracts of the above mentioned drugs and their polyherbal gels were evaluated by excision and incision wound model (*in vivo*) in Sprague Dawley rats. **Results:** The individual gel formulations showed significant reduction in wound size when compared with the untreated group. The rates of wound closure after the application of the gels were compared with the untreated wounds. The polyherbal formulation containing all the extracts was found to be more beneficial when compared to remaining treated groups. Healing under scab and formation of normal epithelial cells were better than other individual formulations. As well as vascular changes, cellular infiltration, and necrosis were found less. **Conclusion:** From the results, it may be concluded that the plants are endowed with significant wound healing activity, thereby justifying its traditional medicinal use in acne vulgaris as a separate entity and in combination. Chemical components such as tannins, flavonoids, saponins, and alkaloids present in the extracts can be responsible for the foresaid activity.

Keywords: Acne, excision model, incision model, wound healing

INTRODUCTION

Wounds are physical injuries that result in opening or break of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part.[1] Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. These cells, through their characteristic "respiratory burst" activity, produce free radicals.[2] Wound related non-phagocytic cells also generate free radicals by involving non-phagocytic nicotinamide adenine dinucleotide phosphate-oxidase mechanism.[3] Thus, the wound site is rich in both oxygen and nitrogen centered reactive species along with their derivatives. The presence of these radicals will result in oxidative stress leading to lipid peroxidation, DNA breakage, enzyme inactivation, and free-radical scavenger enzymes. Evidence for the role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be of therapeutic use in these conditions. Topical applications of compounds with free-radical-scavenging properties in patients have shown to improve significantly wound healing and protect tissues from oxidative damage.[4] In India, medicines based on herbal origin have been the basis of treatment and cure for various diseases and physiological abnormalities under practice such as ayurveda, siddha and Unani. Moreover, Indian folk medicine comprises numerous prescriptions for therapeutic purposes such as healing of wounds, inflammation, skin infections, leprosy, diarrhea, scabies, venereal disease, ulcers, and snake bite. More than 80% of the world's population still depends upon traditional medicines for various skin diseases. Herbal medicines in wound management involve disinfection, debridement

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and providing a moist environment to encourage the establishment of a suitable environment for natural healing process.[5] Research on wound healing agents is one of the developing areas in modern biomedical sciences. Several drugs of plant, mineral, and animal origin are described in the traditional texts of Indian systems of medicine like ayurveda for their healing properties under the term "Vranaropaka." Besides the classical systems of Indian medicine, the folk and the tribal medicine also employ a number of plants and animal products for the treatment of cuts, wounds, and burns. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and human subjects, but the potential of most of the plants remain unexplored.[6]

Inflammation plays a major role in the pathogenesis of acne. As microcomedones form, a lymphocytic infiltrate occur, which triggers inflammation.[7] This tends to elicit follicular keratinocytes further to produce more keratin, as well as stimulating increased sebum production and reducing linoleic acid content in the sebum generated by the sebaceous glands. Most Westernized people have experienced the inflammatory nature of acne vulgaris, given the various red, swollen, tender lesions associated with it, particularly papules, pustules, nodules, and cysts.

Herbs that relieve inflammation could therefore also be useful for limiting or resolving acne. Berberine-containing herbs, besides their antimicrobial action have been shown to be inflammation-modulating.[8] Besides Oregon grape, *Berberis vulgaris* (barberry), *Coptis chinensis* (gold thread), *Hydrastis canadensis* (golden seal), and *Xanthorrhiza simplicissima* (yellow root) all contain berberine and similar alkaloids. Oregon grape has been shown repeatedly to be helpful in clinical trials for patients with psoriasis, another inflammatory skin condition.[9]

Wound healing is a complex process that can be roughly divided into three overlapping phases of inflammatory reaction, proliferation, and remodeling. The inflammatory phase involves vascular responses characterized by blood coagulation and hemostasis as well as cellular events, including infiltration of leukocytes with varied functions in antimicrobial and cytokine release, which initiates the proliferative response for wound repair. During the proliferative phase, there is formation of the epithelium to cover the wound surface with a concomitant growth of granulation tissue to fill the wound space. Granulation tissue formation involves proliferation of fibroblasts, deposition of collagens and other extracellular matrices, and development of new blood vessels. Once the new tissue within the wound is formed, the remodeling phase begins to restore tissue structural integrity and functional competence.[10]

Traditionally, the leaves of *Barleria prionitis* (Acanthaceae), leaves of *Butea monosperma* (Papilionaceae), *Casuarina equisetifolia* bark (Casuarinaceae), *Lagenaria siceraria* fruit (Cucurbitaceae) and *Dalbergia sissoo* bark (Papilionaceae) were used for the treatment of acne.[11] In our past study on these plants showed a promising result in terms of their ability to reduce *Propionibacterium acnes* and *Staphylococcus epidermidis* population, the causative organisms for acne and to act as antioxidants.[12] The present study has been undertaken to assess the wound healing ability of the individual extracts in a gel form and their polyherbal formulation. The ability of these crude drugs to heal wounds can be thought to be helpful in resolving the clinical symptoms associated with acne.

MATERIALS AND METHODS

Plant materials

The leaves of *B. prionitis* (Acanthaceae) and *B. monosperma* (Papilionaceae), *C. Equisetifolia* bark (Casuarinaceae), *L. siceraria* fruit (Cucurbitaceae) and *D. sissoo* bark (Papilionaceae) were collected from Ahmednagar district and authenticated at Botanical survey of India. Voucher specimens of the plants have been deposited at BSI, Pune.

Preparation of extract and preliminary phytochemical screening, the plant materials were dried, coarsely powdered and extracted by continuous hot extraction (soxhlet) method using ethanol (95% v/v). The extracts obtained were concentrated and subjected to phytochemical screening.[13]

Molisch test

The extracts were combined with a small amount of Molisch's reagent (α-naphthol dissolved in ethanol) in a test tube. After mixing, a small amount of concentrated sulfuric acid was slowly added down the sides of the sloping test-tube, without mixing, to form a layer. A positive reaction is indicated by appearance of a purple ring at the interface between the acid and test layers.

Biuret test

The extracts were treated with an equal volume of 1% strong base, i.e., potassium hydroxide followed by a few drops of aqueous copper (II) sulfate. Formation of a purple color confirmed for the presence of proteins.

Ninhydrin test

Three drops of 1% solution of ninhydrin in ethanol were added to 1 ml of the extract solution, and the solution was heated for 5 min in a boiling water bath. The formation of red, blue, or purple color gives a positive result for the presence of proteins.

Dragendorff's test

A volume of 2-3 ml of the solution few drops of Dragendorff's reagent, i.e., potassium bismuth iodide solution was added. An orange-brown precipitate was formed.

Mayer's test

A volume of 2-3 ml of the sample solution few drops of Mayer's reagent, i.e., potassium mercuric iodide solution is added. A white precipitate is formed.

Ferric chloride test

To the extract solution a few drops of 1% $FeCl_3$ was added. Formation of green or blue color indicated the presence of tannins.

Lead acetate test

To the test solution add 10% lead acetate solution. Formation of the white precipitate of lead tannate indicates the presence of tannins.

Shinoda test

To the test solution few magnesium turnings and concentrated hydrochloric acid was added drop-wise. Pink scarlet to green to blue color appears after min, which confirms for the presence of flavonoids.

Liebermann Burchard's test

The extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled, and cooled. Concentrated sulfuric acid was added through the sides of test tube. The formation of brown ring at the junction indicated the presence of steroidal saponins.

Foam test

The extracts were diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. The formation of 1 cm layer of foam indicates the presence of saponins.

Determination of total phenolic content (TPC)

TPC was analyzed by the Folin–Ciocalteu colorimetric method using gallic acid as standard developed by Ragazzi and Veronese with modification and expressed as mg/g gallic acid equivalent (GAE) on dry weight basis.[14] The 25 mg plant extract was dissolved in 10 ml of 50% MeOH:H_2O (1:1), at room temperature and in its 1.0 ml, 1.0 ml of Folin's Reagent (1 N) and 2.0 ml of Na_2CO_3 (20%) were added subsequently. The test mixture was mixed properly on cyclomixer, left at room temperature for 30 min and maintained to 25 ml with water. The absorbance of test mixture was measured at 725 nm. The reported TPC were expressed as GAE mg/g.

Development of the formulation

Literature survey showed that carbomers have many advantages as a gelling agent viz., high viscosity at low concentrations, stability to heat, unaffected by aging, do not support microbial growth, non-toxic and nonirritant, etc.[15,16] Taking into consideration above facts carbomer 940 was taken for the formulation of gel. Eight different gel formulations were prepared containing the extracts at different concentrations.

Wound healing activity

Experimental animals

Wister albino rats (*Rattusnovergicus*), (male) of 4 weeks, weighing between 100 and 150 g were used for wound healing activity. The animals were housed in standard environmental conditions of temperature (31 ± 1°C), humidity (60 ± 0.2%) and 12 h light and 12 h dark cycle. Rats were fed with standard rodent diet and tap water. The approval was obtained from the Animal Ethical Committee of M.C.E Society's Allana College of Pharmacy, Pune.

Excision wound model

The experiments were carried out in accordance with the Animal Ethical Committee after obtaining approval. The backs of the animals were shaved and sterilized with 70% ethanol and tincture Iodine. 5 mm × 5 mm excisions were created by a surgical blade from a predetermined shaved area on the back of each animal. The wounds were left undressed to the open environment, and no local or systemic antimicrobial agents were used. This model was used to monitor the rate of wound contraction. The experimental groups were topically applied with the extract twice daily for consecutive 10 days. The group treated with

Aloe vera gel served as a standard. The wound contractions were measured by tracing paper on the wounded margin and calculated as a percentage reduction in wound area. The actual values were converted into a percentage value taking the size of the wound at time of wounding as 100%. The granulation tissues were monitored on the 4, 8, and 16th post wound days and analyzed for scab formation, necrosis, vascular changes, healing, cellular infiltration, and epithelial cell formation.[17,18] The animals were divided into 10 groups each having five rats (Figure 1).

Group 1: Served as the untreated group.
Group 2: Served as reference standard treated with *Aloe vera* gel
Group 3: Served as test group treated with formulation 1 (*B. prionitis* gel)
Group 4: Served as test group treated with formulation 2 (*B. monosperma* gel)
Group 5: Served as test group treated with formulation 3 (*C. equisetifolia* gel)
Group 6: Served as test group treated with formulation 4 (*D. sissoo* gel)
Group 7: Served as test group treated with formulation 5 (*L. siceraria* gel)
Group 8: Served as test group treated with poly-herbal gel formulation 6 (*B. prionitis* and *C. Equisetifolia* gel)
Group 9: Served as test group treated with polyherbal gel formulation 7 (*C. equisetifolia* and *D. sissoo* gel)
Group 10: Served as test group treated with polyherbal gel formulation 8 (containing all the extracts).

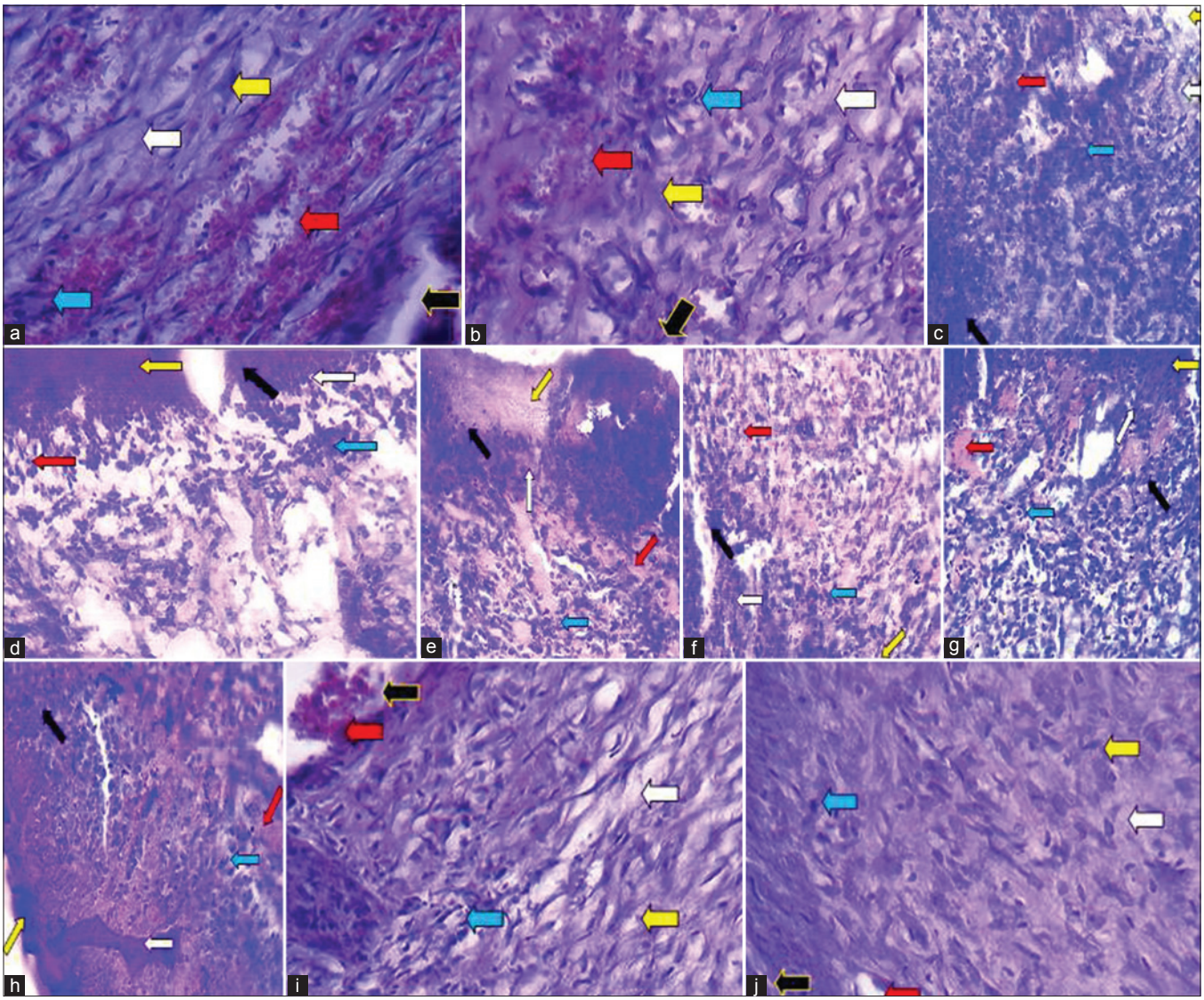

Figure 1: Wound healing activity of the control, standard and the formulations by excision wound model. Scab formation, (yellow arrow), necrosis (black arrow), vascular changes (red arrow), healing (green arrow), cellular infiltration (blue arrow), and epithelial cell formation (white arrow). (a) Control ×400, (b) Standard ×400, (c) Formulation 1 ×400, (d) Formulation 2 ×400, (e) Formulation 3 ×400, (f) Formulation 4 ×400, (g) Formulation 5 ×400, (h) Formulation 6 ×400, (i) Formulation 7 ×400, (j) Formulation 8 ×400.

Incision wound model

Longitudinal para-vertebral incisions of 5 cm in length were made through the entire thickness of the skin and cutaneous muscle with the help of a scalpel. After complete homeostasis, the wounds were closed by means of interrupted sutures placed at equidistant points of 1 cm apart. The sutures were removed on the 8th post wound day, and the topical application of gel formulations and oral administration of the extracts continued. The animals were divided into ten groups each having five rats as in excision wound model[19] (Figure 2).

Histopathological examinations

Wound tissue specimens from untreated and treated rats were taken at day 14 (after treatment). The tissue specimens were taken on 14th day (after treatment), so that the influence of the extracts and reference drugs on skin cells and its components can be evaluated well-instead of

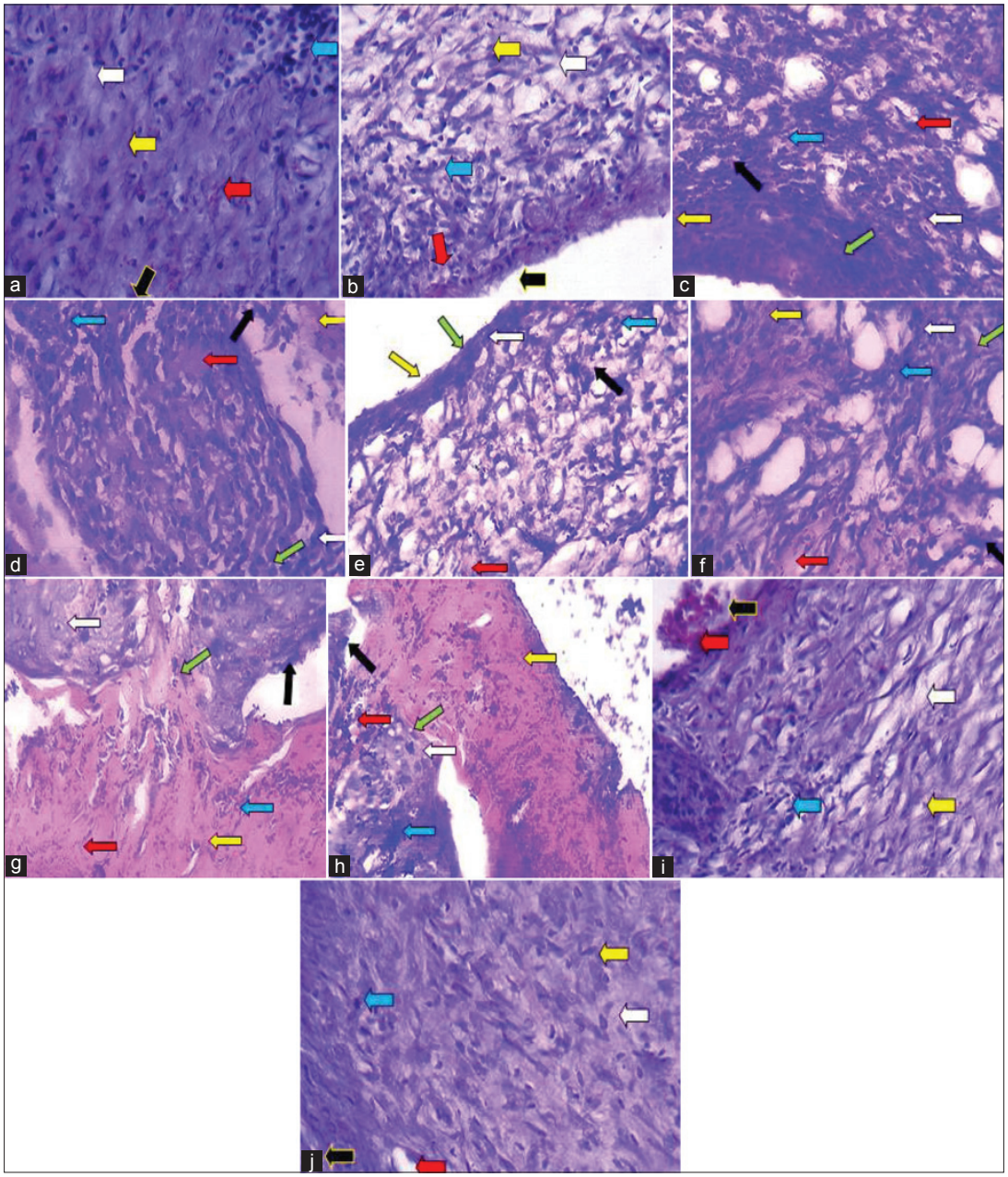

Figure 2: Wound healing activity of the control, standard and the formulations by incision wound model. Scab formation, (yellow arrow), necrosis (black arrow), vascular changes (red arrow), healing (green arrow), cellular infiltration (blue arrow), and epithelial cell formation (white arrow). (a) Control group ×400, (b) Standard ×400, (c) Formulation 1 ×400, (d) Formulation 2 ×400, (e) Formulation 3 ×400, (f) Formulation 4 ×400, (g) Formulation 5 ×400, (h) Formulation 6 ×400, (i) Formulation 7 ×400, (j) Formulation 8 ×400.

the last day of complete wound closure where there will not much difference in the skin tissues and structures. The cross-sectional full-thickness wound scar of about 5 mm thick sections from each group were collected for the histopathological evaluation.[20] Samples were fixed in 10% buffered formalin for 24 h and dehydrated with a solution of the sequence of ethanol-xylene series, processed followed by blocked with paraffin at 40-60°C, and sectioned into 5-6 μm thick sections. The sections were stained with hematoxylin and eosin stain. Collagen deposition was identified by staining with the sections with Van Gieson's stain. Mast cells were stained with toluidine blue.[21]

Measurement of shrinkage of wounds

The measurements of the wound areas of the excision wound model were taken following the initial wound using transparent paper and a permanent marker. The recorded wound areas were measured with graph paper. Progressive decrease in the wound size was monitored periodically.[22]

Measurement of tensile strength

Tensile strength is a measure of the restored tissues resistance to breaking under tension and shows the strength of the healed tissue. The tensile strength was measured after 10th day after wounding. The newly formed tissue including scar was excised, and the tensile strength was measured using a tensiometer (Kruss, GmbH, Germany). The wound breaking strength was measured as the weight of water at the time of wound breaking per area of the specimen.[23]

High performance thin layer chromatography (HPTLC) chemoprofiling of the extracts and the optimized formulation

The HPTLC chemoprofiling of the extracts was done to confirm for the presence of the chemical constituents in the extract as well as the optimized polyherbal formulation.[24] About 100 mg of dry powdered herbal extract was mixed with little quantity of methanol by sonication and then the volume was made up to 10 ml with methanol. Pre-coated silica gel $G_{60}F_{254}$ aluminum plates were used. 10 μl of the

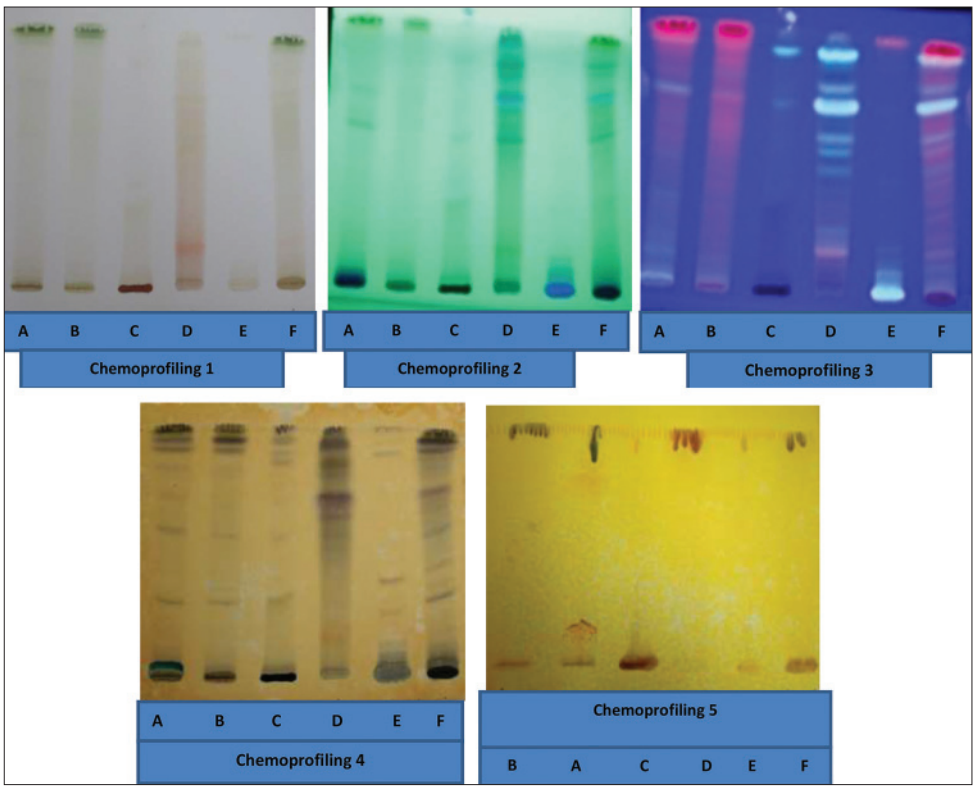

Figure 3: HPTLC chemo profiling showed the presence of tannins in the individual extracts as well as the optimized polyherbal formulation. Chemoprofiling 1 - The HPTLC fingerprinting under visible light, Chemoprofiling 2 - The HPTLC fingerprinting under UV 254 nm, Chemoprofiling 3 - The HPTLC fingerprinting under UV 366 nm, Chemoprofiling 4 - The HPTLC fingerprinting for the confirmation of tannins after spraying with Ferric Chloride reagent and visualized under visible light, Chemoprofiling 5 - The HPTLC fingerprinting for the confirmation of alkaloids after spraying with Dragendorff's reagent and visualized under visible light. A=*Barleria prionitis*, B=*Butea monosperma*, C=*Casuarina equisetifolia*, D=*Dalbergia sissoo*, E=*Lagenaria siceraria* and F=Formulation F8.

Table 1: Formulation of gel

Ingredients	Formulation 1%	Formulation 2%	Formulation 3%	Formulation 4%	Formulation 5%	Formulation 6%	Formulation 7%	Formulation 8%
Carbopol 940	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Propylene glycol	10	10	10	10	10	10	10	10
Propyl paraben	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Methyl paraben	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Triethanolamine	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Distilled water	q.s to make 100	q.s to make 100	q.s to make 100	q.s to make 100	q.s to make 100	q.s to make 100	q.s to make 100	q.s to make 100
B. prionitis extract	1.2	-	-	-	-	1.2	-	1.2
B. monosperma extract	-	1.0	-	-	-	-	-	1.0
C. equisetifolia extract	-	-	0.3	-	-	0.3	0.3	0.3
D. sissoo extract	-	-	-	0.7	-	-	0.7	0.7
L. siceraria extract	-	-	-	-	1.0	-	-	1.0

B. prionitis: Barleria prionitis, B. monosperma: Butea monosperma, C. equisetifolia: Casuarina equisetifolia, D. sissoo: Dalbergia sissoo, L. siceraria: Lagenaria

Table 2: Phytochemical analysis of the plant materials

Secondary metabolite	Test	*B. prionitis*	*B. monosperma*	*C. equisetifolia*	*D. sissoo*	*L. siceraria*
Carbohydrates	Molisch test	+	+	+	+	+
Proteins	Biuret test, Ninhydrin test	-	+	-	-	+
Alkaloids	Dragendroff's test, Mayer's test	+	+	-	-	+
Tannins	FeCl3 test, Lead acetate test	+	+	+	+	-
Flavanoids	Shinoda test	+	+	+	+	-
Steroids	Liebermann Burchard test	-	-	-	+	+
Saponins	Foam test	+	+	-	-	+
TPC (GAE) mg/g		0.8	21.20	45	35.19	85.64

+: Indicates presence of secondary metabolite, -: indicates absence of secondary metabolite. TPC: Total phenolic content, GAE: Gallic acid equivalent,. *B. prionitis: Barleria prionitis, B. monosperma: Butea monosperma, C. equisetifolia: Casuarina equisetifolia, D. sissoo: Dalbergia sissoo, L. siceraria: Lagenaria siceraria*

Table 3: Effect of the formulations on the tensile strength of wound tissues

Wound type	Tensile strength g/mm²
Untreated wound	427.74±4.83
Standard	702.95±3.76*
Formulation 1	572.97±6.74
Formulation 2	563.48±3.99*
Formulation 3	589.44±3.32
Formulation 4	583.87±8.76
Formulation 5	609.87±2.98
Formulation 6	617.36±5.98*
Formulation 7	649.23±1.46*
Formulation 8	687.34±3.86*

*P<0.01

individual ethanolic extracts and the extract of the optimized polyherbal formulation was applied by using HPTLC applicator. Toluene:ethyl acetate:formic acid (5:4:1) was used as the mobile phase. The TLC plates were developed in the developing chamber to a sufficient distance. The detection of spots on TLC plates was carried out by using the spray reagents as 5% ferric chloride and Dragendorff's reagent (Figure 3).

Statistical analysis

For all statistical analyses GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) were used. Data are represented as the mean ± standard error of the mean (N = 5) and analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test. *P < 0.05, **P < 0.01 and ***P < 0.001 were considered as statistically significant in all analyses. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

The preliminary phytochemical screening revealed the presence of tannins, flavonoids, steroids, alkaloids and saponins (Tables 1-7).

DISCUSSION AND CONCLUSION

A number of secondary metabolite compounds isolated from plants have been demonstrated in animal models (*in vivo*) as active principles responsible for facilitating healing of wounds. Some of the most important ones include tannins,[25] alpha-bisabolol and alpha-terpineol,[26] asiaticoside, isolated from *Centella asiatica*.[23] Ethanol extract of the leaves of *Embelia ribes* Burm. (Myrsinaceae) and its

Table 4: Effect of the formulations on wound contraction in excised wounds in rats

Group	Healing with epithelial cell formation	Granulation tissues and scab	Vascular changes	Cellular infiltration	Necrosis
Control	++++	+++	++++	++++	++++
Standard	+++	++	+++	++	++
Formulation 1	+	++	++	+++	++
Formulation 2	+	++	++	++	++
Formulation 3	++	++	++	++	++
Formulation 4	++	++	++	++	+
Formulation 5	++	++	+	+	+
Formulation 6	++	++	++	++	+
Formulation 7	++	++	+	+	+
Formulation 8	+++	+++	+	+	+

+: Damage/active changes up to <25%, ++: Damage/active changes up to <50%, +++: Damage/active changes up to <75%, ++++: Damage/active changes up to more than 75%

Table 5: Summary of wound closures for selected time points

Formulation number	Day				
	1	4	8	12	16
Untreated wound	24.01±102.65	22.56±76.34	21.165±54.32	20.34±28.43	18.49±8.19
Standard	23.04±105.25	11.56±84.26	7.84±58.64**	7.29±31.58	5.76±0.57
Formulation 1	23.23±98.27	20.97±85.29	13.98±49.73	13.54±41.58	11.56±0.52
Formulation 2	22.84±101.65	20.25±75.28	14.51±52.94	12.67±28.91	11.49±1.25
Formulation 3	23.32±111.67	16.16±72.91	12.25±51.92*	9.73±25.61	9.06±1.89
Formulation 4	22.56±100.69	18.92±79.87	12.25±50.37	10.56±22.84***	9.85±1.02
Formulation 5	22.46±113.67	15.68±85.21	9.61±50.98**	9.486±21.29***	8.41±0.98
Formulation 6	20.79±113.28	16.97±73.61	12.96±49.56**	11.9±30.85***	10.36±1.51
Formulation 7	21.9±99.54	16.64±73.51	12.96±51.86**	12.81±29.38***	10.56±1.53
Formulation 8	23.04±101.55	15.28±78.53	8.41±49.56**	7.71±24.85***	6.15±1.46***

Values are mean wound area (mm^2)±SEM for untreated wounds and wounds treated with the different gel formulations *N*=5 rats per group. Data analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. **P*<0.05, **P*<0.01, and ****P*<0.001 were considered statistically significant compared to the untreated wounds. ANOVA: One-way analysis of variance

Table 6: Shrinkage of wounds on 10th day average in (mm) diameter of wound in excision model

Formulation number	Excision	Shrinkage of wounds on 10th day average in (mm) diameter of wound±SEM
Control	5 rats	4.1±42.33
Standard	5 rats	2.8±27.45
Formulation 1	5 rats	3.7±32.98
Formulation 2	5 rats	3.8±31.87
Formulation 3	5 rats	3.5±32.32
Formulation 4	5 rats	3.5±34.17
Formulation 5	5 rats	3.1±30.68
Formulation 6	5 rats	3.6±29.76
Formulation 7	5 rats	3.6±35.37
Formulation 8	5 rats	2.9±28.40

SEM: Standard error of the mean

isolated quinone compound embelin[27] acylated iridoid glycosides from *Scrophularia nodosa*[28] have been reported.

The plant materials taken for screening have reported anti-inflammatory as well as anti-microbial activity, which supports the wound healing process.[29-32] The plant materials have shown for the presence of flavonoid compounds.[33,34] Flavonoids have a good free radical scavenging property as well as lipid peroxidation activity, which also help in the wound healing process.

The antioxidant activities of the individual extracts had been screened in our previous paper and found to be promising.[35] It is likely that the antioxidant property of the extracts could be linked to its wound healing acceleration.[36,37] Topical applications of compounds with antioxidant properties significantly improve wound healing and protect tissues from oxidative damage.[38] Tannins are known to possess antioxidant activity.[39] It could be conceivable that the extracts as a separate entity and in a polyherbal formulation exert their wound healing activity through the tannins since tannins are reported to improve wound healing and protect tissues from oxidative damage.[14] The extracts also exert a significant anti-microbial activity which may be contributed to promote wound healing. In conclusion, the current study revealed that wounds treated with individual gel formulations and their polyherbal formulation extracts significantly accelerate the wound healing process. This wound healing process attributed probably due to the tannins can be considered as a very important factor in reducing acne. Further work is in the process for the isolation of active compounds and their screening for the anti-acne activity.

Table 7: Effect of the formulations on wound contraction in incision wound model in rats

Group	Healing with epithelial cell formation	Granulation tissues and scab	Vascular changes	Cellular infiltration	Necrosis
Control	++++	++++	++++	++++	++
Standard	++	++	++	++++	++
Formulation 1	++	++	++	++	++
Formulation 2	++	++	++	++	+
Formulation 3	++	+++	++	++	+
Formulation 4	++	+++	++	+	+
Formulation 5	++	++	++	++	+
Formulation 6	++	++	++	++	+
Formulation 7	++	++	++	++	+
Formulation 8	+	++	++	++	+

0: No abnormality detected, +: Damage/active changes up to<25%, ++: Damage/active changes up to<50%, +++: Damage/active changes up to<75%, ++++: Damage/active changes up to more than 75%

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Pharmacognosy and Phytochemical Analysis of *Brassica juncea* Seeds

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ABSTRACT

Introduction: *Brassica juncea* is an economically important plant that has been well-known in India for centuries for its medicinal and nutritive values. The broad spectrum of beneficial effects of the seeds perceived with this plant warrants further exploration of *B. juncea* seeds as a potential source for obtaining pharmacologically standardized phytotherapeutics, which could be potentially useful. The objective of the present study was to perform the pharmacognosy of mustards seeds inclusive of qualitative and quantitative phytochemical analysis, fingerprinting by infrared spectroscopy and high performance thin layer chromatography analysis and toxicity assessment *in vitro*. **Methods:** Different sections of seeds were taken and stained with 0.1% phloroglucinol for microscopic examination. The seeds were extracted by 80% alcohol on a rotary shaker to perform phytochemical analysis and fingerprinting. The toxicity assessment of this extract was performed on human dermal fibroblast cells. **Results:** Microscopic examination of seeds showed characteristic features of mustard seeds. The extraction of these seeds by 80% alcohol resulted in IC_{50} value of 103 ± 3 μg/mL for 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl radical scavenging assay. The fingerprinting analysis of this extract indicated probable presence of sinigrin, quercetin, vanillin, catechin, vitamin E and sulfur-containing compounds. This extract exhibited 50% toxicity (IC_{50}) at 1.79 mg/mL. **Conclusion:** The result achieved will be used to assess the therapeutic efficacy of seed extracts for future pharmacological evaluations.

Keywords: Antioxidant, cytotoxicity, Fourier transform infrared spectroscopy, high performance thin layer chromatography, microscopy, phenolics

INTRODUCTION

Brassica juncea Czern. and Coss., also known as Indian mustard, Chinese mustard, oriental mustard, leaf mustard, or mustard green, is a species of mustard family of *Brassicaceae* (cruciferous) plants.[1] *B. juncea* is an economically important plant that has been well known in India for centuries for its medicinal and nutritive values. Various parts of the plants are edible and used in a range of folk medicines and spices. The mustard seeds have been used traditionally for the treatment of muscular rheumatism, inflammatory neuralgic affections, vomiting and dengue.[2] The seeds have also used as folkloric medicine against jaundice in the Jalgoan district of Maharashtra and by the Sugali tribes of the Yerramalais forest of the Eastern Ghats of Andhra Pradesh, India.[3,4] There are various polyherbal formulations of mustard discussed in ayurveda medicine. A decoction of *Moringa oleifera* root (1 in 20) with the addition of bruised mustard seed is useful in doses of 1-2 ounces in ascites due to liver and spleen diseases.[5] A fresh root of *M. oleifera* mixed with mustard seeds and green ginger is used as a counter-irritant and blistering agent.[5] A paste of equal parts mustard, horseradish seeds, hemp seeds and barley mixed with sore buttermilk is a useful application to the scrofulous glands of the neck. According to ayurveda medicine, mustard preparations are mild laxatives, diuretics, and liver-bile stimulators[6] and have been also documented to purge the toxins out of the body.[7] The leaves and seeds of these plants are edible and diverse medicinal uses of seeds are also well-known in other countries. In China, mustard seed is a folk remedy for arthritis, foot ache, lumbago and rheumatism. It is also used in the treatment of tumors; leaves are used in soups for bladder infections, inflammation or haemorrhage. In Korea, the seeds are used for abscesses, colds, lumbago, rheumatism and stomach disorders. The seeds are also reported to be hypoglycemic,[8] antioxidant,[9] anti-diabetic,[10] hyperglycemic,[11] anxiolytic,[12] goiterogenic,[13] and hepatoprotective.[2] The seed paste is used to treat

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backache, arthritis, paralysis, styes, edema of the lungs and liver, aperient, stimulant and emmenagogue.[7,14] The leaves, seeds, and stems have been shown to reduce the severity of asthma and high blood pressure, restore normal sleep attacks and prevent heart attack in patients suffering from atherosclerosis or patterns in women experiencing symptoms of menopause, and reduce the frequency of migraine diabetic heart disease.[15] The hepatoprotective activity of aqueous extract of mustard leaves have been evaluated against carbon tetrachloride-induced hepatic damage in albino rats.[16]

Taken together, these reports strongly suggest the therapeutic potential of seeds against various disorders. However, no definitive evaluation of nature of phytoconstituents involved in their observed effects has yet been made. This inability arises not only from the diverse types of extracts and experimental design used in different studies but also due to variations in the agronomic conditions used in cultivation and harvest. The broad spectrum of beneficial effects of the seeds observed in these studies warrants further exploration of *B. juncea* seeds as a potential source for obtaining pharmacologically standardized phytotherapeutics, that could be potentially useful. To address the lack of literature on standardization, our work focuses on the pharmacognosy of these seeds inclusive of fingerprinting by infrared (IR) spectroscopy and high performance thin layer chromatography (HPTLC) analysis and toxicity assessment *in vitro*.

MATERIALS AND METHODS

Identification and authentication

B. juncea seeds were identified and authenticated at Agharkar Research Institute, Pune, India (Voucher specimen number: S-158).

Macroscopic examination of seeds

The macroscopic characters of the seeds were studied with reference to evaluating organoleptic characteristics.

Microscopic examination of seeds

For microscopic examination, the seeds were taken, and thin sections were cut with a sharp blade. The specimens were stained with pholorglucinol (1% w/v in ethanol) and mounted with glycerol. The photographs of the seeds and its morphology are presented in results.

Determination of ash content and extractive value

Total, water-soluble and acid-insoluble ash contents and water, alcohol and ether soluble extractive values of the powdered seeds was determined as per the standard procedure.[17]

Extraction of phytoconstituents

The extraction of *B. juncea* seeds was carried out by 80% methanol using a rotary shaker for 6 h and the antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was measured.

DPPH radical scavenging activity

The free radical scavenging activity of the extract was measured with stable DPPH in terms of hydrogen donating or radical scavenging activity. 100 μL of DPPH solution (0.36 mm DPPH in methanol) was added to 1 mL extract (100-1000 μg/mL in methanol), vortexed thoroughly and kept in the dark at room temperature for 30 min. Next, the absorbance was measured at 517 nm using ultraviolet-visible (UV-VIS) spectrometer (Perkin Elmer Lambda 25, Perkin Elmer India, Thane, India). Ascorbic acid was used as the positive control. The percentage of inhibition was given by the formula: Percent inhibition (%) = $([A_0-A_1]/A_0) \times 100$, where A_0 is the absorbance of the control solution and A_1 is the absorbance in the presence of the sample and standards.

Qualitative evaluation of phytoextract

Phytochemical analysis of crude extracts were carried out to determine the presence of the various biomolecules using standard procedures.[18] Qualitative tests for tannins, flavonoids, steroids, alkaloids, sugars, proteins, and fats were performed.

Quantitative estimation

Determination of the total flavonoid content

The total flavonoid content was determined by the aluminum trichloride method.[19] Briefly, 1 mL of extract (100-1000 μg/mL) or quercetin standard solution (5-30 μg/mL) was mixed with 1.5 mL distilled water in the test tube, followed by 100 μL aluminum chloride (10%, w/v) and 100 μL potassium acetate (1 M). The reaction mixture was then incubated at room temperature for 45 min and the absorbance was measured at 415 nm by UV-VIS spectrometer. The results of the plant sample were expressed as μg quercetin equivalents/mg extract.

Total phenolic content

The total phenolic content of the extract was determined by the Folin–Ciocalteu reagent method.[19] Briefly, 1 mL of extract or gallic acid (2-10 μg/mL in methanol) was added to 5 mL Folin–Ciocalteu reagent (1:20) and incubated for 5 min at room temperature. Next, 4 mL of sodium carbonate (10% w/v) was added and further incubated for 15 min at room temperature for color development. The absorbance was measured at 765 nm by UV-VIS spectrometer. The amount of total phenolic content was expressed as μg gallic acid equivalent/mg extract.

Determination of sugar and protein content

The sugar content was measured by the 3,5-dinitrosalicylic acid method and the estimation of proteins was carried out by the Lowry method.[15,16]

Fourier transform infrared (FTIR) spectroscopic analysis of plant extract

Approximately, 1 mg of dried extract was pressed into a pellet with 200 mg of potassium bromide and IR spectra were recorded with an accumulation of 45 scans and a resolution of 4/cm on IRPrestige-21 (Shimadzu Corporation, Kyoto, Japan).

Qualitative profiling of extract by TLC

The seed extract was checked by TLC on analytical plates over silica gel 60F254 (Merck and Co., New Jersey, USA). The qualitative analysis for different class of phytoconstituents was carried out by spotting the bands of extract using capillaries and using the mobile phase *n*-butanol:*n*-propanol: water:glacial acetic acid (3:1:1:1) using different spray reagents.

HPTLC fingerprinting

DPPH HPTLC autographic assay

The HPTLC method was used to qualitatively determine the antioxidant activity of extract by DPPH scavenging assay using 0.2% DPPH as a color developer. DPPH is a paramagnetic purple colored compound with an odd electron. The color of the DPPH reagent changes from purple to yellow due to the scavenging of free radicals by antioxidants through donation of hydrogen to form the stable DPPH-H molecule, visible on TLC plates.[18] The method was used for the mobile phase system – toluene:ethyl acetate:glacial acetic acid (4:4:1). Ascorbic acid-the water soluble vitamin and α-tocopherol-the fat soluble vitamin were used as the positive control.

HPTLC marker significant fingerprinting

The HPTLC study was carried out for detecting the presence of vanillin, quercetin and catechin sinigrin. HPTLC fingerprinting was performed at room temperature on aluminum plates pre-coated with silica gel 60F254 (Merck and Co., New Jersey, USA). Solutions of standards and sample were applied to the plates as bands 8.0 mm wide, 10.0 mm apart, and 10.0 mm from the bottom edge of the chromatographic plate using a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 μL Hamilton (India) syringe. Ascending development to a distance of 80 mm was performed using a suitable mobile phase (Table 1) in a Camag glass twin-trough chamber previously saturated with mobile phase vapor for 20 min. After development, the plates were dried and then scanned with a Camag TLC scanner with WINCAT software for quantification.

Cytotoxicity assessment on human dermal fibroblast (HDF) cells

HDF cells were obtained from Scientific Research Center, V. G. Vaze College, Mumbai, India, were grown in 10% fetal bovine serum in Dulbecco's minimal essential medium (DMEM) containing 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in a humidified CO_2 incubator. (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assays of the seed extract on HDF cells were performed. Toxicity toward HDF was assessed with cells

Table 1: HPTLC marker significant fingerprinting of mustard seed extracts

Chemical constituents	Mobile phase	R_f[a]	Quantification (mg/g%)[b]
Vanillin	Toluene:ethyl acetate:glacial acetic acid (4:4:1)	0.62	2.57±0.1
Sinigrin	*n*-butanol: *n*-propanol:glacial acetic acid (3:1:1:1)	0.47	0.7±0.001
Catechin	Toluene:ethyl acetate:glacial acetic acid (4:4:1)	0.15	0.06±0.01
Quercetin	Toluene:ethyl acetate:glacial acetic acid (4:4:1)	0.58	0.13±0.02

[a]Results based on densitometry-HPTLC, [b]values expressed as the mean±standard error.
HPTLC: High performance thin layer chromatography

plated in 96-unit well plates at a density of 5×10^4 cells/well. After adherence, the medium was removed and replaced by serum-free media containing seed extract (0.1-6.4 mg/mL) and incubated for 24 h at 37°C in a humidified CO_2 incubator. Doxorubicin (0.001-10 μM) was used as a positive control. Control cells were incubated with DMEM. Cell viability was determined by measuring the absorbance at 570 and 655 nm. Results were expressed as percentage cellular viability.

RESULTS

Macroscopic and microscopic examination of seeds

The seeds were reddish brown with a smooth texture and approximately 0.9-1 mm in diameter; they had a bitter taste and characteristic pungent smell when crushed. The testa was dark reddish-brown to yellow and minutely pitted. The cells of the outer epidermis of the testa contained mucilage. The embryo was oily and yellow in color, containing two cotyledons folded against their midribs to enclose the radicals (Figure 1).

Physicochemical characterization of seeds

The results obtained for the ash values and extractive values determined by methods described in ayurvedic pharmacopeia, can be used for the quality control purposes for mustard seeds, in various pharmacological interventions. The mean, range and standard error values of ash contents and extractive values of *B. juncea* seeds that resulted from analyses, are summarized in Table 2. The moisture content of the seeds was also determined and found to be <2.1%, which is an important quality control parameter indicating the stability and the susceptibility to bacterial and fungal contamination.

Preliminary characterization of seeds

The preliminary phytochemical study of *B. juncea* seeds was carried out to characterize the chemical constituents present in the extracts following standard procedures. The results indicated the presence of polyphenols such as phenolic acids, flavonoids, alkaloids, and tannins in methanolic extract. The qualitative analysis also indicated the presence of fixed oil content in n-hexane extract of seeds. The calculated values of oils were 30%, similar to reported quantities of 24-35%. Preliminary data were extended to isolate phytoconstituents enriched with antioxidant properties from the seeds.

Extraction of seeds

Mustard seeds were extracted by 80% methanol in water to obtain an antioxidant-rich extract. The yield was calculated

Table 2: Ash and extractive values of seeds of *B. juncea*

Constituent	Content (%)	
	Mean±SE	Range
Total ash	4.56±0.14	4.42-4.7
Water-soluble ash	1.05±0.01	1.04-1.06
Acid-insoluble ash	3.61±0.11	3.5-3.72
Water-soluble extractives	5.21±0.15	5.06-5.36
Alcohol-soluble extractives	9.43±1.2	8.23-10.63
Ether-soluble extractives	25.7±3.2	22.5-27.9

The data is represented as mean±SE, where *n*=3. *B. juncea: Brassica juncea*, SE: Standard error

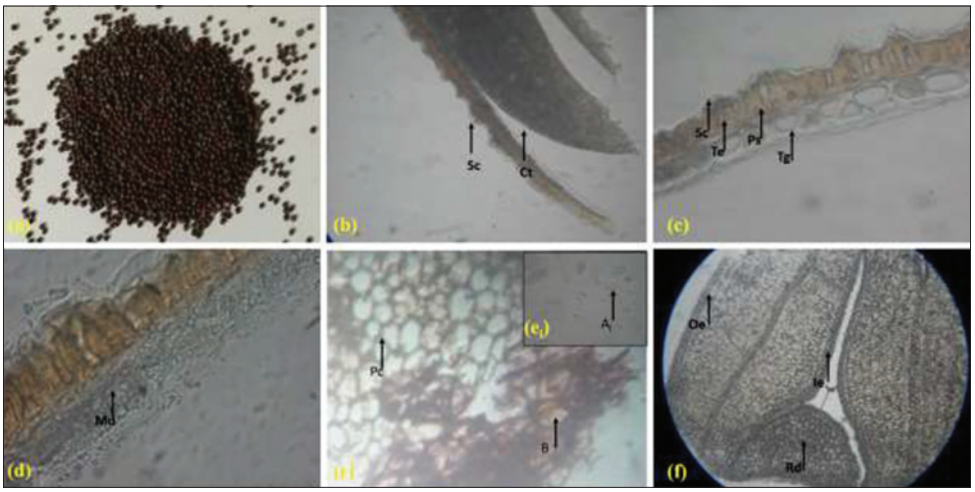

Figure 1: Microscopic examination of mustard seeds stained with phloroglucinol (×100) (full page width). (a) Mustard seeds, (b) seed coat (Sc) and cotyledon (Ct), (c) transverse section of seeds passing through Sc; testa (Te); palisade cells (Ps); and tegmen (Tg), (d) release of mucilage (Mu), (e) parenchyma cells (Pc) and oil globules (B) with the inset (e_i) showing isolated oil globules (A_i), (f) T. S. of seeds showing Ct; outer epidermis (Oe); inner epidermis (Ie) and radical (Rd).

to be of 7.24 ± 0.45% and the IC_{50} value for DPPH radical scavenging capacity was determined to be 103 ± 3 μg/mL.

Quantitative estimations of biomolecules

The flavonoid content of the extract was found to be 4 ± 0.02 μg quercetin equivalent/mg extract (R^2 = 0.9744) and the phenolic content was 107 ± 0.03 μg gallic acid equivalent/mg extract (R^2 = 0.9914). The glucose concentration in the extract was calculated using the equation y = 0.0002x − 0.0033 (R^2 = 0.9695) and was found to be 78.95 ± 6.71 μg N-acetyl glucosamine equivalent/mg extract, whereas the protein content was estimated from the equation y = 0.0028x + 0.013 (R^2 = 0.9961), to be 377.77 ± 00.68 μg bovine serum albumin equivalent/mg extract.

FTIR detected the presence of several functional groups

In FTIR spectroscopy, IR radiation is passed through the extract, from which part of the IR radiation is absorbed by the extract and part of it is transmitted. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the extract representing absorption peaks that correspond to the frequencies of vibrations between the bonds of the atoms present in it. The IR fingerprint of extract also showed presence of multiple peaks, with relatively few however very diagnostic peaks in the region above 2000/cm in contrast the other half contains many peaks with varying shapes and intensities. With the absorption peaks of stretching at 2930, 1665 and 2123.72/cm and bending vibrations at 795.67/cm, BJHAE shows the presence of alkanes, alkenes C=C and alkynes C≡C. BJHAE also showed a broad peak at 3333.14/cm for O-H stretching and 795.67/cm for O-H bending and ring puckering, indicating the presence of alcohols and phenols. Peaks for amines and carboxylic acid O-H bond stretching, C-O-H bending were observed at 1053.18, 2930 and 1426/cm respectively. The sharp peak of 2123.72/cm is a probable indication of presence of isocyanates, isothiocyanates, diimides, azides and ketenes. The spectra also indicated the presence of nitroso and nitro compounds with the peaks at 1514.19, 1514.19 and 1334.8/cm. The peaks at 795.67 and 879.58/cm indicated the presence of sulfane esters, whereas 1053.18/cm an indicative of thiocarbonyl were also present. The extract also showed the peaks for sulfoxide and sulfate at 1053.18 and 1334.8/cm, phosphorous containing compounds phosphine, esters and phosphoramide with the peaks at 1052.18, 926.84, 1053.18 and 1272.11/cm. Oxidized nitrogen is present in the form of oxime and aromatic amine oxides with absorption peaks at 1665.6, 926.84 and 1272.11/cm. Thus FTIR spectroscopy indicated the presence of numerous compounds such as alcohols, phenols, sulfur containing compounds, nitrogen-containing compounds, which are present in plant in abundance and are known to exert various pharmacological effects.

TLC qualitative profile showed the presence of several classes of phytoconstituents

The fingerprinting of extract by TLC was carried out to detect the presence of various class of phytoconstutients that could be present in the extract that are reported to be antioxidants and hepatoprotective. Using different spray reagents, large classes of compounds were detected and observed. A total of seven distinct bands were observed under different spraying conditions with R_f 0.19, 0.26, 0.28, 0.4, 0.43, 0.5 and 0.57. Based on these observations of TLC profile, various classes of phytoconstituents were identified (Table 3).

Fingerprinting analysis of the seed extract

In the study of identification of bioactives, silica plates were used to establish the TLC fingerprint. The characteristic of the extract was observed under UV (254 and 366 nm) and VIS light was compared with the spectra of each component (Table 1). Sinigrin, vanillin, catechin and quercetin were found to be present and were quantified. Of these, vanillin, catechin and quercetin were phenolic acids and reported to be antioxidants. The evidence of antioxidant efficacy was acquired by DPPH HPTLC autographic analysis. The extract showed multiple yellow bands against a purple background when sprayed with DPPH reagent, an indication of antioxidant activity similar to the positive controls: Vitamins C and E (Figure 2).

Non-toxic nature of seed extract on HDF cells

The cytotoxic effect of the seed extract of *B. juncea* was evaluated on HDF cells and it was observed that the extract showed a dose-dependent cytotoxicity (Figure 3) with IC_{50} at 1.79 mg/mL *in vitro*.

DISCUSSION

Brassicaceae vegetables represent an important part of the human diet worldwide and are considered important food crops in China, Japan, India and European countries. The macroscopic and microscopic examination identified the seeds of *B. juncea* by showing typical morphological

Table 3: TLC profile of seed extract indicating the presence of various classes of phytoconstituents

Reagent	Observation			Inference
	Visible	UV short	UV long	
Anisaldehyde-H_2SO_4	Red brown coloration, blue-violet, blue, red	Quenching	Blue, violet, green fluorescence	Essential oils, pigments, triterpenes, saponins
DPPH	Yellow band against purple background	-	-	Antioxidant activity
Dragendorff	Orange-brown	-	Blue	Alkaloids
Ethanolic-H_2SO_4	Brown-black	-	-	Total number of bands
NP-PEG	-	-	Orange, green, blue, blue-green	Bitter drugs, flavonoids, anthracene
Vanillin-H_2SO_4	Lemon yellow, blue, blue-violet, red, yellow brown	-	-	Pungent principle, saponins
Without spraying	-	Quenching	Dark yellow, green, blue, red, dark-blue	Bitter drugs, flavonoids, pigments, pungent principle

DPPH: 2, 2-diphenyl-1-picrylhydrazyl, UV: Ultra violet, TLC: Thin layer chromatography

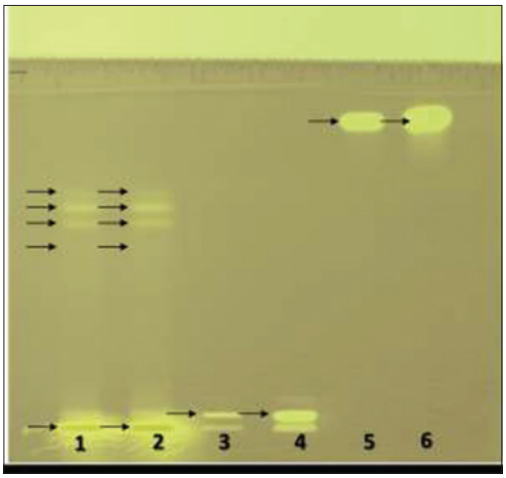

Figure 2: 2,2-diphenyl-1-picrylhydrazyl (DPPH) autographic analysis of mustard seed extract (column width). Key: lane 1 = seed extract (200 μg), lane 2 = seed extract (250 μg/mL), lane 3 = vitamin C (2 μg/mL), lane 4 = vitamin C (5 μg/mL), lane 5 = vitamin E (4 μg/mL), lane 6 = vitamin E (10 μg/mL) when the mobile phase of toluene: ethyl acetate: glacial acetic acid (4:4:1) was used. Antioxidant compounds scavenge the free purple-colored DPPH radicals to the yellow-colored compound. The appearance of yellow color in the and high performance thin layer chromatography plate after developing with 0.2% DPPH reagent indicates antioxidant activity.

characteristics. Furthermore, the total, water soluble and acid-insoluble ash contents of the seeds of *B. juncea* are important indices to illustrate the quality as well as purity of herbal drug. Total ash includes physiological ash, which is derived from the plant tissue itself and non-physiological ash, which is often from environmental contaminations such as sand and soil. *B. juncea*, like other herbal materials, show a variation in the variety and contents of compounds according to differences in growing conditions, such as soil type, climate which may change the ash content depending upon presence or absence of various contaminants thus becoming an important parameter of quality assessment.

B. juncea are known to produce several classes of bioactive phytochemicals including glycosides, flavonoids, phenolic compounds, sterols, triterpene alcohols, glucosinolates (GLSs), proteins and carbohydrates. The available pre-clinical information on this easily cultivable and edible plant strongly suggests that it could be a sustainable source of affordable nutraceuticals or drugs. The beneficial effects of *Brassica* vegetables on health improvement have been partly attributed to their complex mixture of phytochemicals possessing antioxidant activity.[20] Various classes of phytoconstituents from seeds of *B. juncea* were detected via qualitative analysis. The extraction procedure was standardized based on evaluations of DPPH activity as a preliminary tool. Recent reports suggest that cruciferous vegetables act as a good source of natural antioxidants due to their high levels of carotenoids, tocopherols and ascorbic acid.[20] For optimal extraction of antioxidants, 80% methanol was used employing rotary shaker for 6 h, since hydroalcoholic mixtures are the most versatile and widely employed solvent system. Alcohol is at in penetrating cell walls and seed degradation and causes polyphenols to be released from cells; water is a non-toxic solvent with higher polarity. Thus it can solubilize polar compounds to the highest degree, acting as an agent most suited to the extraction of the active principles from plant drugs.[21] This technique translated into a good DPPH scavenging activity (IC_{50} = 103 ± 3 μg/mL) of the extract.

Considering the health benefits, establishing the therapeutic potential of the seeds, phenolics and flavonoids is critical; it was observed that the hydroalcoholic extract that showed promising antioxidant activity characteristic of these phytoconstituents. It has been reported that the flavonoid content of *B. juncea* is not very high, but the spectrum of flavonoids observed for this plant is wider than that of any other plant in the *Brassicaceae* family.[22] Mustard meal has been reported to be a good source of phenolic compounds.

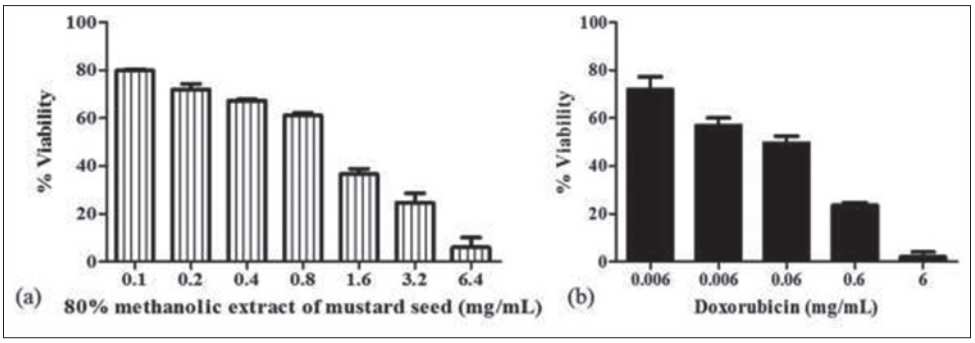

Figure 3: Cytotoxic effect of mustard seed extract on human dermal fibroblast cells (full page width). The cytotoxicity of mustard seeds was assessed by (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay using doxorubicin as a positive control. The dose-dependent reduction in the viability of cells was observed with seed extract and doxorubicin.

More than a dozen phenolic acid conjugates have been reported, and the spectrum of phenolics is also unique and broad.[20] The antioxidant capacity of *Brassica* species has been related to their phenolic profile and content, particularly flavonoids, since phenolic compounds have demonstrated a higher antioxidant activity than vitamins and carotenoids.[20]

Glucosinolates (GLSs) are found in *Brassica* vegetables. Among many such vegetables, the GLS content of *B. juncea* has been reported to be the highest.[23] In fact, the majority of cultivated plants that contain GLSs belong to the family of *Brassicaceae*. In mustard seeds, sinigrin (a GLS) gets converted to allyl isothiocyanate (an organo sulfur compound), releasing glucose under the influence of water by the action of the enzyme myrosinase.[24] The IR spectrum indicated the probable presence of sulfur-containing compounds. Further, the extract also showed the presence of many such functional groups that can result in conjugation reactions within the compounds that can aid in reduction of reactive oxygen species and antioxidant activity of the extract.

Fingerprinting and quantification of phenolics and flavonoids such as quercetin, vanillin, vitamin E and catechin in the hydralcoholic extract was carried out since these phenolics and flavonoids have been reported to be present in mustard seeds. The antioxidant activity of these phytoconstituents has also been well documented.[25-30] Vitamin E competes for scavenging peroxyl radicals much faster than polyunsaturated fatty acids and almost 200 times faster than commercial antioxidant butylated hydroxytoluene[31] since only a small amount of vitamin E can protect a large amount of polyunsaturated fat in the membranes. Quercetin detected in mustard seeds stops propagation of lipid peroxidation, increases glutathione (GSH) levels, antioxidant enzyme function and prevents Ca^{2+} - dependent cell death.[32,33] Catechin is known to reduce lipid peroxidation and increase GSH production.[34] Mustard seeds reported to contain a high content of cysteine residues,[35] may even bind with oxygen electrophiles, further assisting the antioxidant potency. Altogether, we postulate that these phyconstituents contribute to the protective efficacy of extract in the suppression of the elevation of reactive oxygen species generation.

The cytotoxicity assessment of hydroalcoholic extract of *B. juncea* seeds was carried out *in vitro*, since toxicity forms a crucial part in pre-clinical studies resulting in drug failure. In this work, the toxicity assessment was carried out *in vitro* on the cells derived from HDF cells that are most abundant cells in humans; the results revealed that the extract exhibit minimal toxicity *in vitro* that can be further explored for *in vivo* study. Also, it has been reported in a recent work that mustard seeds fed to rats at doses equal to normal human intake do not cause any adverse effects on histopathological parameters.[36] These result can be used to assess the therapeutic efficacy of seed extracts for future pharmacological evaluations.

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Immunomodulatory Effect of Water Soluble Polysaccharides Isolated from *Metroxylon sagu* in Animal Models of Immunosuppression

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ABSTRACT

Aim: This study was aimed to investigate the immunomodulatory activity of water soluble polysaccharides isolated from *Metroxylon sagu* (PSMS) by dilute acid extraction, ethanol precipitation in rats by using three different *in-vivo* experimental models of immunosuppression. **Methodology:** Three models of immunosuppression include metronidazole (MTZ) induced immunosuppression, pyrogallol induced immunosuppression and Ethanol-induced immunosuppression. Immunological indices like humoral antibody titer values, cellular immune response, percent change in phagocytosis, serum immunoglobulins were estimated. Histopathology of spleen was done in all control and treated groups. The doses of 500 and 250 mg/kg of PSMS were administered orally to evaluate the immunomodulatory activity. **Results:** Though PSMS was demonstrated to have immunostimulatory activity in almost all three models of immunosuppression, PSMS was found to be more efficacious against ethanol-induced immunosuppression when compared with pyrogallol induced immunosuppression and MTZ induced immunosuppression. However, dose-dependent improvement in immunological indices was evident in all three models. **Conclusion:** In summary, water soluble polysaccharides isolated from *M. sagu* stimulate the immunity in the animal models of immunosuppression.

Keywords: Immunomodulation, immunosuppression, *Metroxylon sagu,* oxidative stress, polysaccharides

INTRODUCTION

Immune system dysfunction is responsible for various diseases such as arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer, and infectious diseases. Chemotherapeutic agents available today have mainly immunosuppressive activity and most of them are cytotoxic and exerts a variety of side-effects. Medicinal plants and their active components as a source of immunomodulatory agents are gaining importance.[1] Many herbs such as *Centella asiatica, Azadirachta indica, Phyllanthus debelis, Asparagus racemosus*, and *Chenopodium ambrosioides* have been shown to alter the immune function and to possess a wide array of immunomodulatory effects.[2] True sago palm is one of genus *Metroxylon* belongs to family arecaceae or palmae. The plant accumulates a huge amount of starch in its stem, very often more than 100 kg/plant. It contains mainly 80% starch, 16% water, 2% nitrogenous substance, and very little ash. Its utilization includes a wide range of consumption types, containing staple food, noodle-making, confectionery and fuel alcohol.[3] Traditionally, stem sap of *M. sagu* is applied to the forehead to ease headaches. Starch derived from the plant trunk is mixed with water and drunk to treat diarrhea and stomach pains. Starch paste is also applied on to burn. The Leaf is used to cover fresh or infected sores until they heal. Liquid starch is given to newborn to treat enlarged spleen.[4] Since there is no scientific data on the immunomodulatory activity of this plant, this study was undertaken to evaluate the immunomodulatory activity of water soluble polysaccharides isolated from the *M. sagu* in rats.

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MATERIALS AND METHODS

Drugs and chemicals

Metronidazole (MTZ) was gifted by Zydus Cadila Health Care Limited (Hyderabad, India), India. Pyrogallol

was a gifted by Hi Media Laboratories Private Limited (Hyderabad, India), Levamisole was gifted by Leo Bio-Care Private Limited (Hyderabad, India). All the chemicals were purchased from local agents, and these are of analytical grade.

Plant material

The seeds of *M. sagu* belongs to the family Arecaceae were collected at the local areas of Anantapur district (Andhra Pradesh, India) in winter season in the month of November 2010 and were authenticated by Dr. J. Ravindra Reddy and voucher specimen (Raghavendra Institute of Pharmaceutical Education and Research-05/11) was preserved in the departmental herbarium (Pharmacognosy and Ethnopharmacology Division) for future reference.

Extraction of polysaccharides

M. sagu seeds were collected and pulverized into a coarse powder and used for extraction of polysaccharides. About 160 g of the seeds of *M. sagu* was allowed to stand in 1 L of 0.1 N HCl for overnight at room temperature. The extract was filtered through an atypical woman's nylon sock. Later, the filtrate was neutralized with 1 N NaOH and polysaccharides were precipitated with three volumes of ethanol. After centrifugation for 30 min, the precipitate was re-dissolved in distilled water. The pH of the suspension was adjusted to 2.0 with 1 N HCl, and $CaCl_2$ was added to the final concentration of 2 M. The resulting precipitate was removed by centrifugation and the supernatant was treated with three volumes of ethanol. The ethanol precipitation was repeated twice and the precipitate was re-dissolved in distilled water, dialyzed at 4°C against water for 48 h, and then freeze-dried.[5]

Experimental animals

Wistar rats of 180-200 g were used to carry out the immunomodulatory activity. The animals had free access to standard commercial diet and water *ad libitum* and were housed in cages under standard laboratory conditions, i.e., 12:12 h light/dark cycle at 25 ± 2°C. The experimental protocol was approved by the Institutional Animal Ethics Committee (Protocol number is PIPER/IAEC/05/2011 and all experiments were carried out in compliance with CPCSEA guidelines. (878/ac/05/CPCSEA/003/2011).

Acute toxicity study of the polysaccharides isolated from *Metroxylon sagu* (PSMS)

The PSMS was subjected to acute toxicity studies to determine the dose for the *in-vivo* studies. Wistar mice of either sex were selected randomly and divided into six groups (n = 6). The animals were fasted overnight and the PSMS at a dose of 200, 400, 800, 1000, 2000, and 4000 mg/kg body weight, were given orally to the mice. The animals were observed carefully for any sign of morbidity, mortality, and behavioral changes immediately after being dosed at 4 h and at 24 h intervals and twice daily for the subsequent 7 days.

Assessment of immunomodulatory activity

For the assessment of immunomodulatory activity, we selected three models like MTZ induced immunosuppression,[6] Pyrogallol induced immunosuppression[7] and ethanol induced immunosuppression.[8] Animals were treated with polysaccharides isolated from *M. sagu* (PSMS) at a dose of 250 and 500 mg/kg. Experimental design for immunological studies was shown in Table 1.

Immunological studies

Hemagglutinating antibody (HA) titer

Blood was withdrawn from the jugular vein of a sheep red blood cells (SRBCs) were preserved in Elsevier solution. It was then suspended in phosphate buffered saline for further use. All rats were anti-genically challenged twice with SRBC (0.025×10^9 cells/100 g, i.p.).

Blood samples were collected from the retro-orbital plexus and rat serum was used for determination of hemagglutination titer. The blood samples were centrifuged to collect serum and equal volume of individual serum samples of each group was pooled. Sera were serially diluted (in doubling dilutions) in phosphate buffered saline (PBS) and placed in the wells of a U-shape 96-Microtiter plates. Aliquots (25 µl) of two-fold diluted sera in PBS were challenged with 25 µl of 1% v/v SRBCs suspension and mixed. After mixing, the plates were incubated at 37°C for 1 h and examined for hemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody title.[9]

Delayed-type hypersensitivity (DTH) response

The rats were challenged by injection of 0.5×10^9 cells SRBCs in right hind footpad. The increase in the paw volume induced by injection of SRBC (0.025×10^9 cells) in the sub plantar region of right hind paw was assessed after 48 h of this challenge. The mean percent increase in paw volume was considered as DTH reaction and considered as an index of cell-mediated immunity. The volume of left hind paw injected similarly with phosphate buffered saline, served as control.[7]

Table 1: Experimental design for the immunological studies

Animal model	Treatment and test doses used	Timings of antigen challenge for humoral response	Estimation day for humoral response	Timings of antigen challenge for cellular response	Estimation day for cellular response
MTZ induced immunosuppression model	PSMS 250 mg/kg and 500 mg/kg	On 8th day	On 13th day	On 13th day	On 15th day
Pyrogallol induced immunosuppression model	PSMS 250 mg/kg and 500 mg/kg	On 7th and 13th day	On 13th and 20th day	On 20th day	On 22th day
Ethanol induced immunosuppression model	PSMS 250 mg/kg and 500 mg/kg	On 14th and 20th day	On 20th and 27th day	On 27th day	On 29th day

PSMS: Polysaccharides isolated from Metroxylon sagu, MTZ: Metronidazole

Carbon clearance test

In all the three models, Wistar rats were treated with the drug or vehicle as per treatment schedule. After 3 h of the last dose of the drug, animals were injected 0.1 ml of carbon ink (camel fountain pen ink) suspension (1.6% v/v in 1% gelatin, in saline) through the tail vein. Blood samples were withdrawn (in 0.15% w/v disodium ethylenediaminetetraacetic acid [EDTA] solution) from a retro-orbital vein at intervals of 0 and 15 min after injection. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate (Na_2CO_3) solution and the absorbance of this solution was determined at 660 nm, taking 0.1% Na_2CO_3 solution as a blank. The percentage increase in phagocytic index was calculated.[10]

Serum immunoglobulins (Igs)

Serum Igs like immunoglobulin G (IgG) was estimated[11] using the kit (Quantia) on the last day of the treatment, i.e., 15th day in case of MTZ induced immunosuppression, 22nd day in case of pyrogallol induced immunosuppression and 28th day in the case of ethanol-induced immunosuppression, respectively.

In-vivo anti-oxidant parameters

In-vivo antioxidant parameters such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (RSH), and lipid peroxidase (LPO) were estimated on the last day of the treatment, i.e., 14th day in case of MTZ induced immunosuppression, 22nd day in case of pyrogallol induced immunosuppression and 28th day in the case of ethanol-induced immunosuppression, respectively.

Preparation of erythrocyte lysate

The blood samples were withdrawn into EDTA containing eppendorf tubes on the last day of the treatment from the retro-orbital venous plexus of rats. Then, these eppendorf tubes were subjected to centrifugation at 8000 RPM for 15 min. The supernatant was discarded, and erythrocyte lysate was prepared from the sediment.

SOD

It was estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 µl of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA, and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by spectrophotometer. The activity of SOD is expressed as units/mg protein.[12]

CAT

A volume of 50 µl of the lysate was added to a cuvette containing 2 ml of phosphate buffer (pH 7.0) and 1 ml of 30 mM H_2O_2 CAT activity was measured at 240 nm for 1 min using a spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M/cm was used to determine the CAT activity. One unit of activity is equal to 1 mmol of H_2O_2 degraded per min and is expressed as units/mg of protein.[13]

RSH

To 1 ml of sample, 1 ml of 10% trichloroacetic acid (TCA) was added. The precipitated fraction was centrifuged, and 2 ml 5,5'-dithiobis(2-nitrobenzoic acid) was added to 0.5 ml supernatant. The final volume was made up to 3 ml with phosphate buffer. The color developed and the optical density was measured at 412 nm using spectrophotometer.[14]

LPO

About 2 ml of sample was mixed with 2 ml of 20% TCA and kept on ice for 15 min. The precipitate was separated by centrifugation and 2 ml of samples of clear supernatant solution were mixed with 2 ml aq. 0.67% TBA solution. This mixture was heated on a boiling water bath for 10 min. It was cooled in ice for 5 min and absorbance was measured spectrophotometrically at 535 nm. The values were expressed as nanomoles of malondialdehyde formed per milligram of protein values are normalized to protein content of tissues.[15]

Histopathological examination

All groups of rats except PSMS 250 mg/kg lower dose were sacrificed by cervical dislocation on the 15th day in case of MTZ induced immunosuppression, 22nd day in case of pyrogallol induced immunosuppression and 28th day in the case of ethanol-induced immunosuppression, respectively. Spleen of each rat was then collected, fixed in 10% formalin and sectioned. However, histopathological studies were not carried out for a lower dose (PSMS 250 mg/kg). Hence, histopathological changes were presented (Figures 1-7) in the spleen were observed under light microscope.[6]

Statistical analysis

The results were expressed as mean ± standard error of the mean. The differences were compared using one-way analysis of variance followed by Tukey's test.

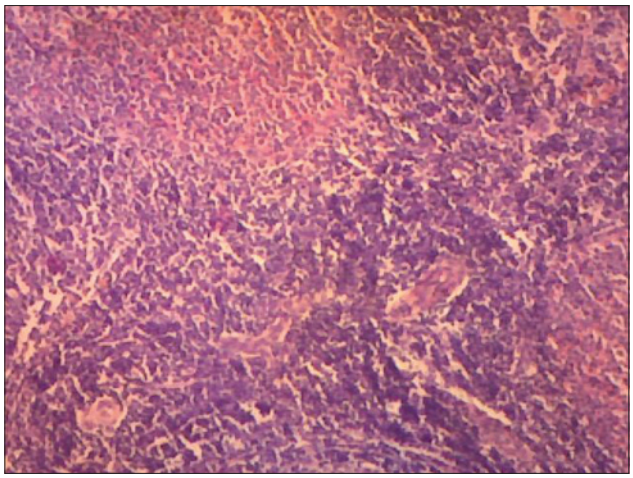

Figure 1: Normal spleen showing both white pulp and red pulp.

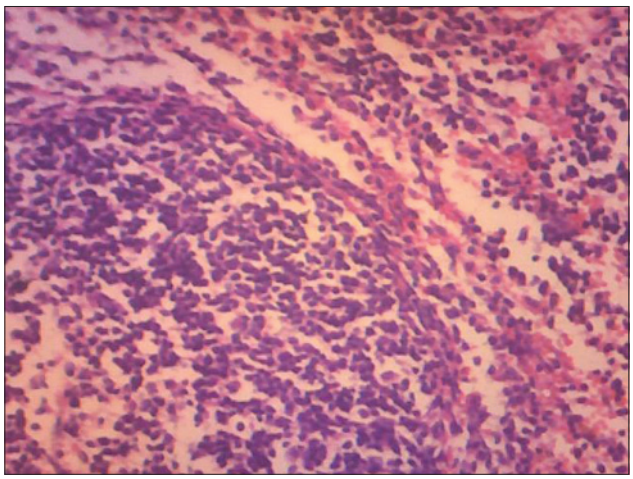

Figure 2: Metronidazole treated animals showing congested red pulp and atrophy of white pulp.

RESULTS

Acute oral toxicity

It was observed that water soluble PSMS was not lethal even at the dose of 4000 mg/kg, body weight following oral administration in mice.

Effect of PSMS on immunological parameters

Animals treated with MTZ, pyrogallol and ethanol alone showed significant ($P < 0.05$) decrease in the immunological parameters such as a humoral immune response, cellular immune response, carbon clearance test, and serum Igs, respectively.

Animals treated with PSMS showed significant ($P < 0.05$) and dose-dependent increase in the immunological parameters such as humoral immune response, cellular immune response, carbon clearance test, and serum Igs in all three models (Tables 2-4).

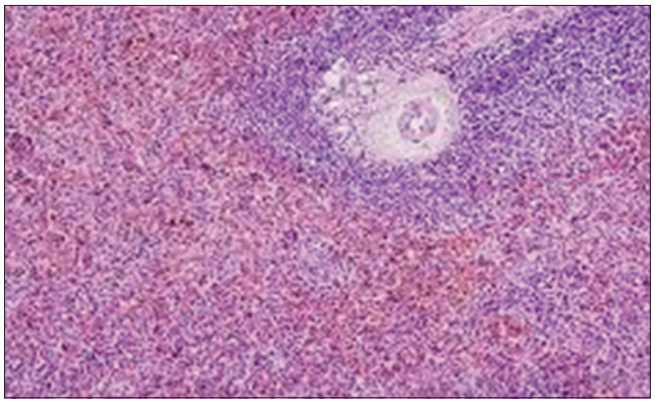

Figure 3: Metronidazole + polysaccharides isolated from Metroxylon sagu treated animals showing normal looking red pulp and white.

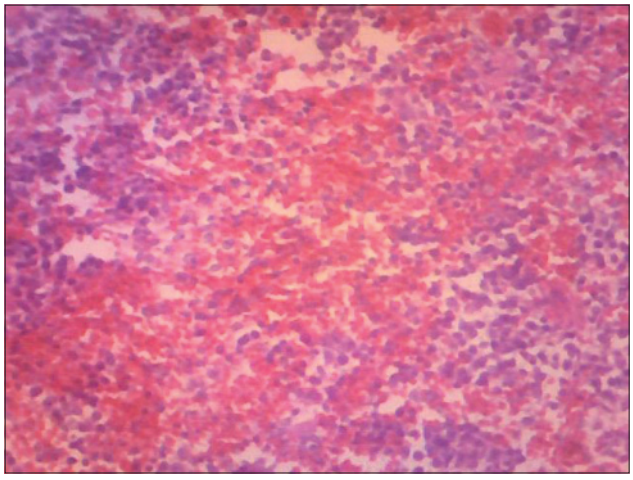

Figure 4: Pyrogallol treated animals showing congestion of red pulp.

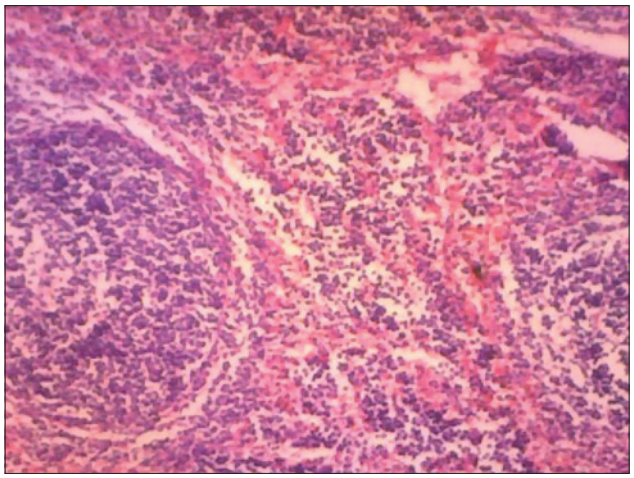

Figure 5: Pyrogallol + polysaccharides isolated from *Metroxylon sagu* treated animals showing normal looking red pulp and white pulp.

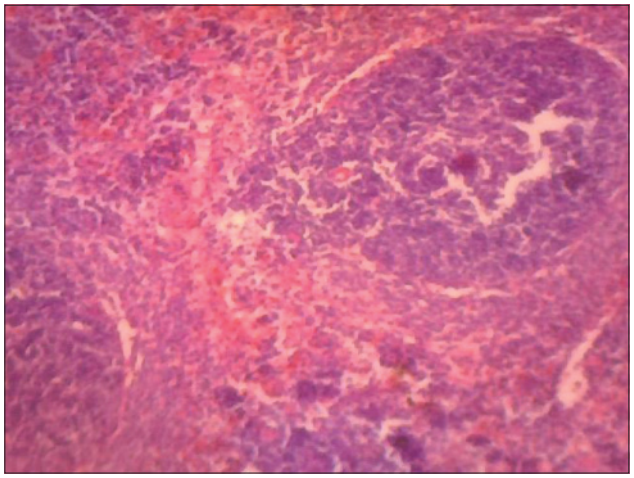

Figure 6: Ethanol treated animals showing congestion of white pulp and red pulp.

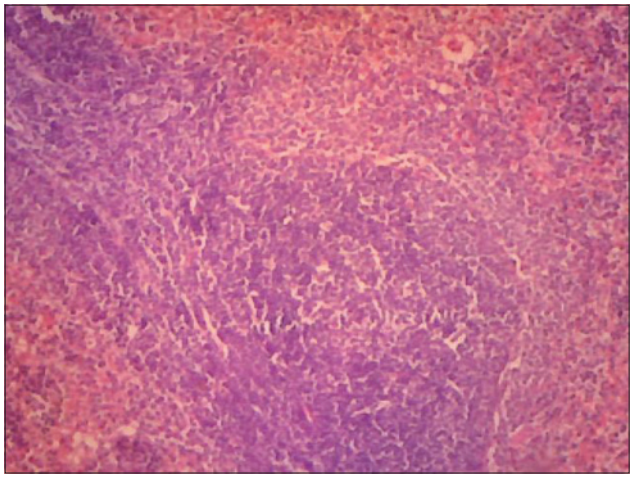

Figure 7: Ethanol + polysaccharides isolated from *Metroxylon sagu* treated animals showing normal looking white pulp and red pulp.

Effect of PSMS on *in-vivo* oxidative and antioxidant parameters

Animals treated with MTZ, pyrogallol and ethanol alone showed significant ($P < 0.05$) decrease in the SOD, CAT, RSH levels and significant ($P < 0.05$) increase in the lipid peroxidation levels. Animals treated with PSMS showed significant ($P < 0.05$) and dose-dependent increase in the SOD, CAT, RSH levels and a significant decrease in the lipid peroxidation levels in all three models (Tables 5-7).

Effect of PSMS on histopathology of spleen tissue

Light microscopic examinations of spleen showed remarkable differences between MTZ, pyrogallol and ethanol-treated animals and PSMS treated animals. Atrophy in the spleen white pulp and congestion of red pulp was observed in control animals but not in PSMS treated animals (Figures 1-7).

DISCUSSION

Despite the overt use of MTZ as an antibacterial and anti-parasitic in humans, there is little information about its potential influence on the immune system cellularity and function. MTZ has been shown to induce suppression in the bone marrow, a primary lymphoid organ, and to affect male fertility. Furthermore, it has been observed that MTZ induces DNA single-strand breaks in the lymphocytes of patients on standard doses of the drug; therefore, toxicity in the peripheral lymphoid organs is suspected.[6]

Pyrogallol is toxic to the biological system, and its toxicity is attributed to its ability to generate free radicals. The literature has documented several evidences of the vulnerability of the immune system to the free radical-induced oxidative stress, which indicate that the cellular and humoral components of the immune system are particularly sensitive to increased levels of reactive oxygen species, which may cause premature immunosenescence.[8]

The administration of ethanol, over a period of four weeks, not only impaired the immune responses, but also produced oxidative stress, in rats. Since the immunotoxic effects of ethanol may be due to oxidative stress. The literature has documented free radical generation during the metabolism of ethanol. The level of the markers of oxidative stress, observed in ethanol-treated rats substantiates the possibility of extensive generation of free radicals.[16]

Table 2: Effect of PSMS on immune response in MTZ induced immunosuppression in rats

Group	HA titer values	Cellular immune response (percentage change in paw volume)	Percent change in phagocytosis	Serum immuno globulins (mg/dL)
Normal	9.8±0.8	34±1.1	88±2.4	437±11.2
Disease control	2.3±0.06$^{\#\#}$	16±4.6$^{\#\#}$	48±1.1$^{\#\#}$	276±9.5$^{\#}$
Levimasole (standard)	9.2±0.8***	31±2.3*	76±0.8***	398±16.41*
PSMS (250 mg/kg)	6.1±0.7***	22.5±2.5**	60.4±3.1***	342±8.7**
PSMS (500 mg/kg)	9.3±0.4***	38±3.5**	77±0.8***	412±7.2**

All values are expressed as mean±SEM, *n*=6 in each group, $^{\#\#}P$<0.001 when compared to normal, $^{\#}P$<0.01 when compared to normal, ***P<0.001 when compared to control, **P<0.01 when compared to control, *P<0.1 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, HA: Humoral antibody, MTZ: Metronidazole

Table 3: Effect of PSMS on immune response in pyrogallol induced immunosuppression in rats

Group	HA titer values		Cellular immune response (percentage change in paw volume)	Percent change in phagocytosis	Serum immunoglobulins (mg/dL)
	Primary	Secondary			
Normal	10.5±0.5	10.7±1.4	34±1.1	88±2.4	437±11
Disease control	1.1±0.06$^{\#\#}$	5.0±0.5$^{\#\#}$	9±0.1$^{\#\#}$	53±0.6$^{\#\#}$	293±11$^{\#}$
Levimasole (standard)	9.6±0.8***	9.9±0.7***	25±3.3*	86±0.3***	422±7.5*
PSMS (250 mg/kg)	4.3±0.6***	7.8±0.9***	18.5±2.3***	61.4±1.7***	335±10**
PSMS (500 mg/kg)	9.9±0.7***	10.3±0.5***	30±1.1**	82±1.7***	400±16**

All values are expressed as mean±SEM, *n*=6 in each group, $^{\#\#}P$<0.001 when compared to normal, $^{\#}P$<0.01 when compared to normal, ***P<0.001 when compared with control, **P<0.01 when compared to control, *P<0.1 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, HA: Humoral antibody

Table 4: Effect of PSMS on immune response in ethanol induced immunosuppression in rats

Group	HA titre values		Cellular immune response (percentage change in paw volume)	Percent change in phagocytosis	Serum immunoglobulins (mg/dl)
	Primary	Secondary			
Normal	11.0±0.8	11.5±0.4	34±1.1	88±2.4	437±11
Disease control	2.9±0.03$^{\#}$	8.1±0.3$^{\#}$	8±0.6$^{\#}$	57±0.6$^{\#}$	326±3.6$^{\#}$
Levimasole (standard)	10.0±0.6*	10.2±3.4*	30±0.1*	87±1.4*	447±4.7*
PSMS (250 mg/kg)	6.6±1.8*	9.8±0.5*	21.6±3.0*	75±3.4*	398±4.1*
PSMS (500 mg/kg)	10.9±2.0*	11.7±1.4*	33±0.1*	93±1.5*	455±0.5*

All values are expressed as mean±SEM, *n*=6 in each group, $^{\#}P$<0.001 when compared to normal, *P<0.001 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, HA: Humoral antibody

Table 5: Effect of PSMS on oxidants and anti-oxidant enzymes in MTZ induced immunosuppression in rats

Group	Superoxide dismutase (units/mg protein)	Catalase (units/mg protein)	Glutathione µmol DTNB (conjugated/g Hb)	LPO (nmMDA/g Hb)
Normal	34±0.6	286±6.0	5.7±0.1	96±0.6
Disease control	18±1.4$^{\#}$	160±2.8$^{\#\#}$	3.4±0.05$^{\#\#}$	179±1.7$^{\#\#}$
Levimasole (standard)	33±0.5**	213±3.3**	4.2±0.1*	118±1.5***
PSMS (250 mg/kg)	26±1.9**	182±4.5**	4.1±0.1*	149±4.3***
PSMS (500 mg/kg)	37±2.5**	213±3.3**	4.3±0.2*	122±1.1***

All values are expressed as mean±SEM, *n*=6 in each group, $^{\#\#}P$<0.001when compared to normal, $^{\#}P$<0.01 when compared to normal, ***P<0.001 when compared to control, **P<0.01 when compared to control, *P<0.1 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, LPO: Lipid peroxidation, DTNB: 5,5′-dithiobis (2-nitrobenzoic acid), Hb: Hemoglobin, MDA: Malondialdehyde, MTZ: Metronidazole

Table 6: Effect of PSMS on oxidants and anti-oxidant enzymes in pyrogallol induced immunosuppression in rats

Group	Superoxide dismutase (units/mg protein)	Catalase (units/mg protein)	Glutathione µmol DTNB (Conjugated/g Hb)	LPO (nmMDA/g Hb)
Normal	34±0.6	286±6.0	5.7±0.1	96±0.6
Disease control	21±1.2$^{\#}$	195±2.8$^{\#\#}$	4.4±0.1$^{\#}$	163±2.5$^{\#\#}$
Levimasole (standard)	39±1.0***	240±2.0***	5.2±0.1*	97±1.4***
PSMS (250 mg/kg)	30±2.5***	225±3.2***	4.8±0.08*	136±4.6***
PSMS (500 mg/kg)	40±1.1***	246±4.6***	5.5±0.1**	101±1.0***

All values are expressed as mean±SEM, *n*=6 in each group, $^{\#\#}P$<0.001 when compared to normal, $^{\#}P$<0.01 when compared to normal, ***P<0.001 when compared to control, **P<0.01 when compared to control, *P<0.05 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, LPO: Lipid peroxidation, DTNB: 5,5′-dithiobis (2-nitrobenzoic acid), Hb: Hemoglobin, MDA: Malondialdehyde

Table 7: Effect of PSMS on oxidants and anti-oxidant enzymes in ethanol induced immunosuppression in rats

Group	Superoxide dismutase (units/mg protein)	Catalase (units/mg protein)	Glutathione µmol DTNB (Conjugated/g Hb)	LPO (nmMDA/g Hb)
Normal	34±0.6	286±6.0	5.7±0.1	96±0.6
Disease control	25±1.0$^{\#}$	198±1.1$^{\#\#}$	4.1±0.1$^{\#\#}$	154±1.1$^{\#\#}$
Levimasole (standard)	40±0.3**	261±1.3**	5.2±0.1*	93±1.5**
PSMS (250 mg/kg)	29±2.3**	254±5.6**	4.9±0.09**	123±3.2**
PSMS (500 mg/kg)	43±1.4**	279±4.7**	5.7±0.1**	87±2.9**

All values are expressed as mean±SEM, n=6 in each group, $^{\#\#}P$<0.001when compared to normal, $^{\#}P$<0.01 when compared to normal, $^{**}P$<0.001 when compared to control, $^{*}P$<0.05 when compared to control. PSMS: Polysaccharides isolated from *Metroxylon sagu*, SEM: Standard error of the mean, LPO: Lipid peroxidation, DTNB: 5,5′-dithiobis (2-nitrobenzoic acid), Hb: Hemoglobin, MDA: Malondialdehyde

Phagocytosis is the process in which phagocytes, ingests and removes microorganisms, malignant cells, inorganic particles, and cellular debris.[17] The carbon clearance test was done to evaluate the effect of drugs on the reticuloendothelial system (RES). The RES is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream.

When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation.[18] Water soluble PSMS isolated from *M. sagu* 500 mg/kg showed a remarkable augmentation in the phagocytic index by exhibiting increase in clearance rate of carbon by the cells of the RES, it is speculated that it might be due to increase in the activity of the RES by prior treatment of animals with water soluble PSMS isolated from *M. sagu*.

IgG and IgM antibodies are involved in the complement activation, opsonization, neutralization of toxins, etc.[17] The successive oral treatment of water soluble PSMS isolated from *M. sagu* showed a significant response in antibody production against SRBC compared to the control group. The enhancement of antibody responsiveness to SRBC in mice, in this study, indicated the enhanced responsiveness of macrophages and B lymphocyte subsets involved in the antibody synthesis. Therefore, augmentation of the humoral immune response to SRBCs by water soluble PSMS isolated from *M. sagu*, as evidenced by increase in the antibody titer in rats indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis.[19] The high values of HA titer obtained in the case of water soluble PSMS isolated from *M. sagu* indicated that the immunostimulation was achieved through humoral immunity.

Cell-mediated immunity involves effectors mechanisms carried out by T lymphocytes and their products (lymphokines).[17] In immune inflammatory DTH reaction, macrophages and Th1 cells plays a major role. This reaction requires a specific antigenic substance, which will release cytokines by activation with T-lymphocytes.[20] Here, SRBC was used as the antigenic substance, which elicits the hypersensitivity reaction in mice. Therefore, increase in DTH reaction in rats in response to T cell-dependent antigen revealed the stimulatory effect of water soluble PSMS isolated from *M. sagu* on T cells in all the three models of immunosuppression.

CONCLUSION

The results of the study indicated that water soluble PSMS has remarkable and dose-dependent immunostimulatory activity against three models. The order of immunostimulant activity of PSMS against three models is ethanol-induced immunosuppression model > pyrogallol induced immunosuppression model >MTZ induced immunosuppression model.

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Development and Antifungal Evaluation of Cinnamaldehyde Containing Silver Nanoparticles against *Candida albicans*

Sir,

The use of engineered silver nanoparticles (AgNPs) has got tremendous attention in all of the medical applications.[1] Recent studies explored antifungal potential of AgNPs as well cinnamaldehyde against *Candida albicans*. AgNPs may exert an antifungal activity by disrupting the structure of the cell membrane and inhibiting the normal budding process due to the destruction of the membrane integrity.[2,3] Cinnamaldehyde is pale yellow, viscous liquid occurs naturally in the bark of cinnamon trees and other species of the genus *Cinnamomum*. The essential oil of cinnamon bark is about 90% cinnamaldehyde. Exact mode of action of cinnamaldehyde is not clear, but it acts on multiple sites, especially on cell membranes and endomembranous structures of the fungal cell.[4] Candidiasis, mainly caused by *C. albicans,* is acute, subacute or chronic infection and the most common fungal disease often affects the skin, mucous membranes, even causes visceral or systemic infection. Again as a part of treatment of Candidiasis, large doses of antibiotics, hormones, and immunosuppressant can be cause of serious life-threatening consequences. Hence, major market demand is natural alternative to toxic antifungal agents and with same objective, the present investigation is exploring synergistic effects of two potent antifungal agents AgNPs and cinnamaldehyde in the form of cinnamaldehyde containing AgNPs against *C. albicans*. Antifungal effects of samples like cinnamaldehyde alone, AgNPs alone and cinnamaldehyde containing AgNPs on *C. albicans* found to be promising.

Bark of *Cinnamomum zeylanicum* (Figure 1) was purchased from the local market of Amravati City (Maharashtra) and authenticated by Head, Botany Department, Government Vidarbha Institute of Science and Humanities, Amravati, Maharashtra. The bark pieces were dried and coarsely powdered in a cutter-mill. The powdered material was stored in an air tight container until further use. Powdered *Cinnamon* bark was used to extract *Cinnamon* oil (30 ml) by Clevenger apparatus. To isolate cinnamaldehyde from oil, 5-10 ml of dichloromethane was added to oil, mixed slowly, followed by allowed to separate, further drain off. Same procedure repeated two more times and all of the dichloromethane layers were combined. Dichloromethane solution dried by adding sodium sulfate until it is free flowing and then evaporated on the rotovap. A clear, slightly yellow liquid cinnamaldehyde (21.5 ml) with a strong odor of *Cinnamon* was obtained. Isolated cinnamaldehyde is confirmed by high performance thin layer chromatography (stationary phase: silica Gel F^{254} and mobile phase: toluene-ethyl acetate [9:1]) and Fourier transform infrared spectroscopy as shown in Figure 1. The infrared spectroscopy spectrum displayed characteristic bands corresponding to aromatic CH bonds, between 3000/cm; to CH alkenes, between 3020/cm and 3080/cm; to C=C, between 1640/cm and 1680/cm; and to the aldehyde C=O group between 1690/cm and 1760/cm.

AgNPs are prepared by chemical reduction method where ethanol is used in the presence of poly (N-vinylpyrrolidone) polyvinylpyrrolidone (PVP) as a stabilizing agent. 10 ml of 1% (w/v) ethanolic solution of PVP and 0.2 ml of 0.1 M $AgNO_3$ were taken in a 25 ml closed conical flask and placed in a microwave oven (RASA, model: R-259) that was operated at the 100% power of 700 W and frequency 2450 MHz for 5 s.[5] Rapid microwave heating and agitation gives monodispersed particles. The colorless solution instantaneously turned to the characteristic pale yellow colour, indicating the formation of AgNPs. Excess PVP was removed by centrifugation and multiple washing. Solution of purified AgNPs (0.5 ml in 10 ml methanol) then incubated with cinnamaldehyde (1 ml) for 10 min to get cinnamaldehyde containing AgNPs.

The AgNPs were characterized (Figure 2) in a ultraviolet–visible spectrum (UV-VIS) spectrophotometer (Model no - UV 1700 Shimadzu) to know the kinetic behavior of Ag nanoparticles. Jeol JSM-6480 LV Scanning Electron Microscope (SEM) machine were used to characterize mean particle size and morphology of nanoparticles. A laser diffraction method (Nano Sight (NTA) Ver. 2.2 Build 0366, Malvern Aimil Instruments) with a multiple scattering technique has been used to determine the particle size distribution of the liquid.[6] Solution of AgNPs showed maximum absorbance in UV-VIS spectroscopic analysis at 422 nm. From particle size and SEM image analysis, AgNPs

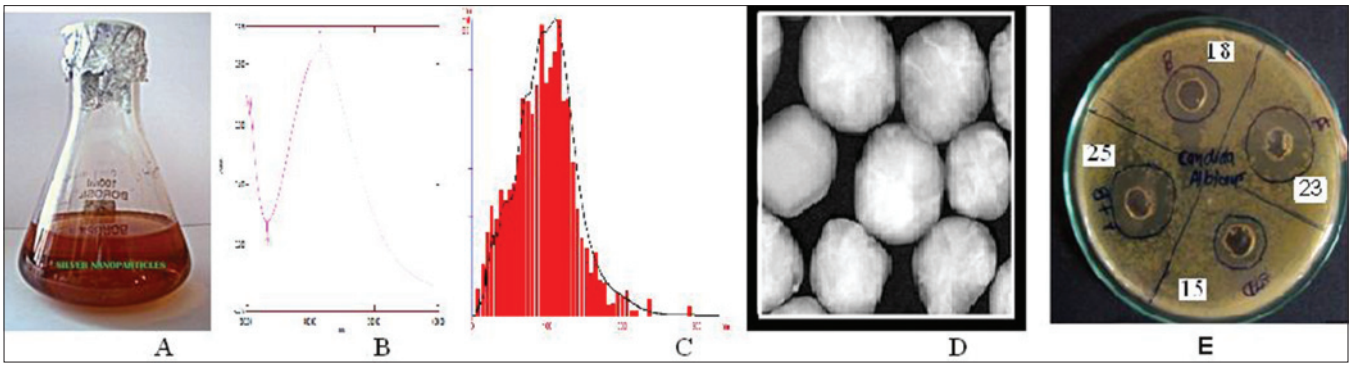

Figure 1: (a) *Cinnamon zeylanicum* bark and cinnamon oil isolation using Clevenger apparatus. (b) Fourier transform infrared spectroscopy spectrum of isolated cinnamaldehyde. (c) High performance thin layer chromatography (stationary phase:silica gel F^{254} and mobile phase:toluene- ethyl acetate [9:1]) chromatogram of isolated cinnamaldehyde.

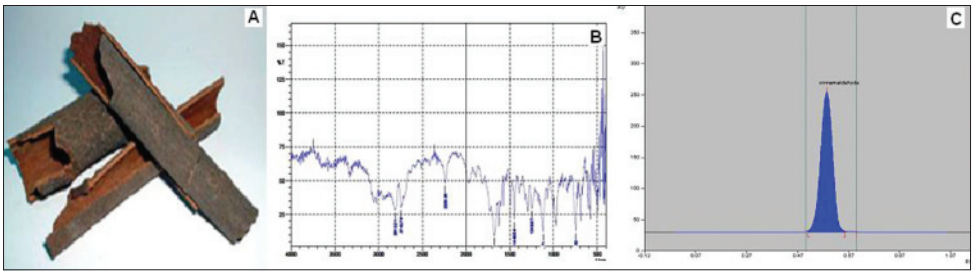

Figure 2: Characterization of silver nanoparticles (AgNPs). (a) Color change due to AgNP formation. (b) Ultraviolet (UV)-visible spectrum showing UVmax at 420 nm. (c) Particle size concentration and particle size relative intensity. (d) Scanning electron microscope image of cinnamaldehyde containing AgNPs. (e) Antifungal activity of all samples against *Candida albicans* disc diffusion method. Where, A = Cinnamaldehyde (70 ppm); B = AgNPs, (50 ppm); A + B = Cinnamaldehyde (70 ppm) AgNPs (50 ppm); saturation transfer difference = Standard (fluconazole-70 ppm) showing zone of inhibition 23 mm, 18 mm, 25 mm and 15 mm respectively.

was found to be spherical in shape, range of particle size between 10 and 100 nm and average size was 85 nm.

Antifungal potential of both cinnamaldehyde and AgNPs was tested against *C. albicans* by disc diffusion method. Fungal cultures were diluted suitably with the sterile saline solution to bring the count to about 1×10^8/ml. The 2 mg/ml solution in dimethyl sulfoxide of all the fractions was prepared. Fluconazole (2 mg/ml) was used as positive control. Antifungal activity of all samples was evaluated by disc diffusion method using Sabouraud dextrose agar. The fungi inoculate were homogenously seeded onto the 90 mm petri dishes containing 20 ml cooled molten medium. After 15 min, three wells were bored in each petri dish at equal distance. Two drops of each sample and standard solution were added to the respective wells and named accordingly. The plates were left at room temperature for about 1 h to allow the extract to diffuse from the disc into the medium, and were then incubated at 30°C for 48 h. After incubation the diameter of zone of inhibitions (in mm) were measured. The complete triplicate antifungal analysis was carried out under strict aseptic conditions. From the study, it is confirmed that cinnamaldehyde containg AgNP (A + B) exists synergistic effect against *C. albicans* fungi. All samples that is., Cinnamaldehyde (A), AgNPs (B) and combinations of both were found more potent than antifungal fluconazole. It is also confirmed that synergistic effect exists when cinnamaldehyde (A) is combined with AgNPs (B).

CONCLUSION

In the present work, spherical, monodispersed cinnamaldehyde containg AgNPs are synthesized by chemical method. Our findings attempts to highlight that cinnamaldehyde containing nanoparticles have promising synergistic antifungal effect against *C. albicans* and have the potential to be an effective alternative to conventional antifungal agents for future therapies in *Candida*-associated complexities, but deserving further investigation for clinical applications.

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