Chemical Composition and Biological Activities of the Essential Oil of *Plectranthus caninus* Roth

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ABSTRACT

The essential oil from the aerial parts of *Plectranthus caninus* Roth (Lamiaceae), obtained by hydro-distillation was analyzed by gas chromatography/mass spectrometry (GC/MS) and evaluated for antimicrobial, free radical scavenging and anti-inflammatory activities. Thirty-four compounds representing 91.02% of the total oil were identified. The major constituents of the oil were camphor (22.36%) and α-thujene (14.48%). The oil was tested against 21 bacterial and 4 fungal strains using disc diffusion method and found to be active against a broad spectrum of pathogens including Gram-positive and Gram-negative bacteria as well as some fungal strains. The minimum inhibitory concentrations (MICs) of the oil against the bacterial strains tested ranged from 10 to 400 µg/ml, and from 800 to 1000 µg/mL against the fungal strains employed. The *in vitro* antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging assay. The oil reduced DPPH in a concentration dependent manner with an EC_{50} value of 3.5 µl/ml. The *in vivo* anti-inflammatory activity was evaluated on the basis of inhibition of carrageenan-induced mouse hind paw oedema whereby doses of 200 and 300 mg/kg were found to inhibit significantly increase in paw volume during the late phase of inflammation. The study provides evidence for the broad-spectrum antimicrobial, significant antioxidant and anti-inflammatory effect of *Plectranthus caninus* essential oil, a possible explanation for the traditional use of the plant in the treatment of cold, teeth and gum disorders which may be related to microbial infections and inflammation.

Key words: Anti-inflammatory activity, antimicrobial activity, camphor, free radical scavenging activity, hydro-distillation.

INTRODUCTION

In recent years, an upsurge of interest in the use of natural substances as phytomedicines has resulted in a more thorough investigation of plant resources. Aromatic plants and their essential oils, used since antiquity in folk medicine and for the preservation of food, are known sources of natural secondary metabolites having biological activity such as antimicrobial, antioxidant and anti-inflammatory action among many others.[1] *Plectranthus* is a genus rich in essential oils. It contains about 300 species found in Tropical Africa, Asia and Australia with a diversity of ethnobotanical uses.[2, 3]

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Plectranthus caninus Roth (Lamiaceae) is a branched, succulent, and pubescent annual herb that attains a height of 15-40 cm. It grows in dry stony ground, rocky areas and road sides at an altitude between 1000-2600 m above sea level.[4] In Eastern Africa, it is used to treat teeth and gum disorders. [3] In northern part of Ethiopia *P. caninus* is known by its vernacular name "Endifdif" and it is traditionally used as anthelmintic and for the treatment of common cold. In Kenya, old men use its leaves as snuff, a habit that can become addictive.[3] Perusal of literature has revealed the isolation of phenolics[5] and diterpenes[6] from the leaves of *P. caninus*. The diuretic[5, 7], cytotoxic and antitumor promoting[5] activities of the plant are also reported.

As a continuation of our research on essential oil bearing plants of Ethiopia,[8-15] we report herein the chemical composition, antimicrobial, radical scavenging and anti-inflammatory properties of the essential oil distilled from the aerial parts of *P. caninus*.

MATERIALS AND METHODS

Plant material

The aerial part of *P. caninus* was collected in June 2007 from in and around the city of Addis Ababa, Ethiopia. The authenticity of the plant material was confirmed by Ato Melaku Wondaferash, the National Herbarium, Department of Biology, Addis Ababa University, where a voucher specimen was deposited.

Essential oil distillation

The fresh aerial part of *P. caninus* was extracted by hydrodistillation of 1 kg of the plant material using a Clevenger-type apparatus for 4 h. The oil obtained was stored in a sealed amber coloured vial in a refrigerator at -10°C until use.

Gas chromatography/Mass spectrometry analysis

Qualitative and quantitative GC/MS analyses were carried out on a Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced to an HP 5989B mass spectrometer. Separation was done on a 25 m x 0.25 mm HP5-MS capillary column coated with 0.50 µm 5% phenyl 95% methylpolysiloxane. Temperature programming was set at 70-250°C, at a rate of 3°C/min. The carrier gas used was helium at a constant flow rate of 1.9 ml/min. Injector and interface temperature were adjusted to 250°C and 280°C, respectively. EI mass spectra were recorded at 70 eV ionization voltage (source temperature 250°C). Compounds were identified by mass spectral comparison with a commercial database (Wiley8 and NISTO5 mass spectral library) and the laboratory's own database. Spectral data were compared with linear retention indices published in the literature.[16- 18]

Bacterial strains

The oil was tested against the following Gram-negative bacterial strains: *Escherichia coli* K99, *E. coli* K88, *E. coli* 306, *E. coli* LT37, *E. coli* 872, *E. coli* ROW 7/12, *E. coli* 3:37C, *E. coli* CD/99/1, *Salmonella typhi* Ty2, *Shigella boydii* D13629, *S. dysentery* 1, *S. dysentery* 8, *S. flexneri* Type 6, *S. soneii* 1, *Vibrio cholerae* 1313, *V. cholerae* 293, *V. cholerae* 1315, *V. cholerae* 85. The Gram- positive bacterial strains used were *Bacillus pumilus* 82, and *B. subtilis* ATCC 6633 and *Staphylococcus aureus* ML 267.

All the bacterial strains were procured from the Department of Pharmaceutical Technology, Jadavpur University; Central Drug Laboratory, Kolkata and Institute of Microbial Technology, Chandigarh, India. The strains were first checked for purity on the basis of standard microbiological, cultural and biochemical tests and then used for sensitivity testing towards the essential oil.

Fungal strains

Antifungal activity testing was carried out on the following fungal pathogens: *Aspergillus niger* ATCC 6275, *Candida albicans* ATCC 10231, *Penicillium funiculosum* NCTC 287 and *P. notatum* ATCC 11625. All the fungal strains were procured in lyophilized state from the Institute of Microbial Technology (IMT), Chandigarh, India and preserved in the laboratory.

Antibacterial activity evaluation

The minimum inhibitory concentration (MIC) of the oil was determined by checkerboard technique using nutrient agar medium.[19-21] The zones of inhibition produced by the essential oil were determined and compared with that of pure ciprofloxacin by disc diffusion technique.[21, 22] Two sets of dilutions (100 µg/ml) whereby each of the oil dissolved in dimethyl sulphoxide (DMSO) and ciprofloxacin dissolved in sterile distilled water were prepared in sterile McCartney bottles. Sterile nutrient agar plates were prepared and incubated at 37°C for 24 h to check for any possible contamination. Three sterile filter paper discs (What man no. 1) of 6 mm diameter were soaked in the same dilution of the essential oil (each 6 mm disc was shown to absorb 25 µl of oil solution or reference drug stock solution in order to be saturated) and placed in an appropriate position on the plate, marked as quadrant at the back of the Petri dishes. The Petri dishes were incubated at 37°C for 24 h and the diameters of the zones of inhibition were measured in mm. A similar procedure was adopted for the pure ciprofloxacin and the corresponding zone diameters were compared.

Determination of mode of antibacterial action of the oil

The bacteria were allowed to grow in nutrient broth overnight, from which 2 ml were added to 4 ml of sterile nutrient broth and incubated for further 2 h at 37°C so that the culture attained a logarithmic phase of growth. The essential oil was then added at a higher concentration than the MIC value for that particular strain. The number of colony forming units (CFU/ml) were determined by the method described by Miles and Misra at an interval of 2 h up to 6 h and then after 18 h. [23]

Antifungal activity evaluation

The antifungal activity was first evaluated by estimation of MIC of the oil against the fungal pathogens followed

by the disc diffusion study with griseofulvin as a reference standard. To determine whether the oil was fungistatic or fungicidal, plugs from the zone of inhibition were taken out and re-incubated in fresh media that were then examined for their growth after 96 h incubation at 25°C.[24, 25]

Free-radical scavenging activity

The free-radical scavenging activity of the essential oil was determined with 2,2-diphenyl-1-picrylhydrazil (DPPH) assay [26] The samples were diluted with methanol to prepare 172, 333, 585 and 667 µl of the essential oil/ml solutions. Five ml of 0.004% DPPH solution was pipetted into each test tube followed by the addition 50 µl of sample solutions. After the mixture was incubated at 37 ºC for 30 min in the dark, the absorbance was measured against blank at 517 nm using Jenway 6505 UV/VIS spectrophotometer. The percent of DPPH scavenged was calculated using the following equation:

Percent of DPPH scavenged (%) = ((Ao – A)/Ao) × 100

where, Ao is absorbance of the control (containing all reagents except the test compound) and A is absorbance of the tested sample at 517 nm. Tests were carried out in triplicate and vitamin C was used as a positive control.

The EC_{50} value (effective concentration that decreases the initial DPPH absorbance by 50%) of the oil was calculated from the linear regression plots of concentration vs. percent of DPPH scavenged.

Anti-inflammatory activity

Animals

Swiss Albino mice of either sex with body weight 25-30 g were used for the experiment. The animals were procured from the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa. Animal quarters were maintained at a temperature of 22 ± 2°C with 12-h light/12-h dark cycle. They were fed on a standard mouse diet. All animals were fasted overnight before oral dosing; tap water was available *ad libitum*.

In vivo antiinflammatory activity testing

In vivo anti-inflammatory activity was evaluated on the basis of inhibition of carrageenan-induced mouse hind paw oedema as described by Marroquin-Segura *et al*.[27] The essential oil 100, 200 or 300 mg/kg diluted with 1% Tween 20, indomethacin (10 mg/kg) and the vehicle were given orally to the experiment, reference and control groups, respectively. Animals of either sex were used in the experiment and each group was composed of six mice (three male and three female). The oedema inducing agent, i.e. 0.05 ml of 1% carrageenan in normal saline was then injected into the plantar surface of the left hind paw 30 min after oral administration of the test substances. The volumes of injected paws were measured before, and 60, 120, 180 and 240 min after injection of carrageenan using Ugo Basile plethysmometer (Italy, model 7140). The increase in paw volume, i.e. inflammation (%I) was calculated according to the equation given by Delporte *et al*.:[28]

$$\%I = \frac{Vf - Vi}{Vi} \times 100$$

where Vf and Vi are the final and initial paw volumes of each animal, respectively. The mean %I was then calculated and a curve of mean %I versus time was plotted.

In addition, the anti-inflammatory effect (%A) was calculated according to the formula given below[28] and data were presented as mean ± standard error of the mean (SEM).

$$\%A = \frac{\%Ic - \%Ie}{\%Ic}$$

where $\%I_c$ and I_c are the inflammation values reached in control and experimental groups, respectively.

Statistical analysis

Data were expressed as mean± SEM, and statistical significance was determined via one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test with $p < 0.05$ considered to be significant.

RESULTS AND DISCUSSION

Chemical composition analysis

The percentage yield of the essential oil of the fresh aerial part *P. caninus* obtained by hydrodistllation was 0.3% (v/w). The oil was yellowish with unpleasant scent having specific gravity, refractive index and optical rotation ($[\alpha]_D^{22}CHCl_3$) of 0.92, 1.48 and +21.56°, respectively. Qualitative and quantitative analyses carried out by GC/MS identified 34 compounds representing 91.02% of the total oil of which five were unknown. Two of the unknowns were shown to be oxidized sesquiterpenes. The results of GC/MS analysis are summarized in Table 1.

The major constituents of the essential oil of *P. caninus* were camphor (a bicyclic monoterpene ketone, 22.36%) and α-thujene (a bicyclic monoterpene hydrocarbon, 14.48%). About 80% of the components in the essential oil were monoterpens of which 37% were oxygenated while the remaining were non-oxygenated monoterpenes. Eleven percent of the total oil was composed of sesequiterpenes,

Table 1: Composition of the essential oil of the aerial part of *Plectranthus caninus*.

Compound	Retention index HP5-MS column	Content (Area % method, GC-MS analysis)
α-Thujene	932	14.48
α-Pinene	940	1.06
Thuja-2,4(10)-diene	945	5.44
Camphene	949	1.53
Sabinene	974	1.30
1-Octen-3-ol	980	0.46
β-Myrcene	993	0.35
α-Terpinene	1016	6.69
p-Cymene	1026	7.99
Thujol	1028	0.84
Limonene	1030	2.22
1,8-Cineol	1040	0.30
γ-Terpinene	1061	1.94
Unknown1, m/z 166,151,123,119,109,91	1085	1.05
Fenchone	1090	8.49
Linalool	1101	0.68
Unknown 2, m/z 166,151,134,123,119,108,91	1113	0.33
Unknown 3, m/z 152,134,123,119,109,91,81	1115	0.50
Camphor	1150	22.36
Terpinen-4-ol	1180	4.03
p-Cymene-8-ol	1187	0.36
α-Terpineol	1193	0.36
α-Copaene	1380	0.36
trans-Caryophyllene	1425	1.61
α-Caryophyllene (α-humulene)	1460	1.61
Germacrene D	1487	0.50
β-Selinene	1492	1.72
γ-Cadinene	1519	0.37
Δ-Cadinene	1528	0.64
Caryophyllene oxide	1591	1.25
Oxidised sesquiterpene, m/z 220,205,177,138,109,96,67	1617	0.91
epi-α-Cadinol	1647	1.38
α-Cadinol	1661	0.70
Oxidised sesquiterpene, m/z 220,202,177,159,122,109,91,79	1693	0.30
Total (identified)		**91.02**

6.81% being non-oxygenated. 1-Octen-3-ol (0.46%) was detected as the only non-terpenoidal component of the oil.

Antimicrobial activity

Antimicrobial studies were conducted on 21 bacterial and 4 fungal strains. The antibacterial susceptibility screening revealed that the essential oil exhibits a broad-spectrum activity against both Gram-positive and Gram-negative bacteria and also against a few fungal strains (Table 2).

The MIC of the oil against the bacterial strains tested ranged from 10 to 400 µg/ml and from 800 to 1000 µg/ml against the fungal strains employed. Comparison of the effect of the oil against the various test bacterial strains indicated that it was significantly active on almost all strains of *E. coli, Shigella* spp, *S. aureus* and *V. cholerae* with an MIC value of 25 µg/ml. *Shigella flexneri* Type 6 was the most susceptible organism to the oil against which the oil exhibited an excellent activity (MIC=10 µg/ml). The effect of the oil against *S. typhi* and the two *Bacillus* species used in this study was significantly lower. As shown in Table 2, the oil exhibited an average inhibition of 94.5% on the growth of *E. coli* strains and 93% on *V. cholerae* when compared with that of ciprofloxacin. As there was no evidence of growth after incubation of the bacteria in the presence of the oil at a concentration level above the respective MIC values, the mode of action of the oil was considered to be bactericidal.

The oil did also demonstrate maximum fungal inhibitory potency against *C. albicans* with an MIC value of 800 µg/ml. At a concentration of 2000 µg/ml, the average inhibitory activity of the oil against *C. albicans* was 85.6% when compared with that of griseofulvin. Both *Pencillum* spp used in this study were inhibited at a concentration of 1000 µg/ml. The mode of action of the oil was proved to be fungicidal since incubation of plugs taken from the zones

Table 2: Antibacterial and antifunga activities of the essential oil of the aerial part of *Plectranthus caninus*.

Bacterial strain	MIC (µg/ml)	Mean zone of inhibition in mm of 200 µg/ml drug or oil*		% Activity of oil compared with that of ciprofloxacin
	oil	oil	ciprofloxacin	
Escherichia coli K99	25	15.0	16.0	93.7
E.coli K88	25	15.5	16.5	93.9
E.coli 306	25	15.5	16.5	93.9
E.coli LT37	25	15.0	16.0	93.7
E.coli 872	25	15.5	16.0	96.9
E.coli ROW 7/12	50	15.0	16.5	93.9
E.coli 3:37C	25	15.0	15.5	93.5
E.coli CD/99/1	25	16.5	17.0	96.7
Salmonella typhi Ty2	100	14.0	15.0	93.3
Shigella dysentery 1	50	16.0	20.5	78.0
S. dysentery 8	50	16.5	21.0	78.6
S. soneii 1	25	17.0	19.5	87.2
S. boydii D13629	25	18.0	20.0	91.0
S. flexneri Type 6	10	18.5	20.5	90.0
Staphylococcus aureus ML 267	25	15.0	18.0	83.3
Bacillus pumilus 82	200	12.0	18.5	64.8
B. subtilis ATCC 6633	400	11.5	18.0	63.8
Vibrio cholerae 1313	25	16.5	17.0	97.0
V. cholerae 293	25	16.5	17.5	94.3
V. cholerae 131	25	16.5	18.0	91.7
V. cholerae 85	25	16.0	18.0	88.9
Fungal strain	**MIC in µg/ml**	**Mean zone of inhibition in mm of 2000 µg/ml drug or oil***		**% Activity of oil compared with that of griseofulvin**
	oil	**oil**	**griseofulvin**	
Candida albicans ATCC 10231	800	13.5	16.0	84.4
Aspergillus niger ATCC 6275	1000	12.5	15.0	83.3
Penicillium notatum ATCC 1625	1000	12.0	13.5	88.9
P. funiculosum NCTC 287	1000	12.0	14.0	85.7

* Mean zone of inhibition in mm including 6 mm diameter of disc (n=3).

of inhibition after 96 h did not result in the growth of any of the fungal strains tested.[24, 25]

The present study, therefore, provides evidence for the broad-spectrum antimicrobial effect of *P. caninus* essential oil. This in turn may explain the traditional use of the plant for the treatment of common cold. The antibacterial and antifungal effects of the oil could be attributed to the presence of camphor, the major constituent of the oil, and other minor components such as α-pinene and terpinen-4-ol which have been reported to be effective against bacteria and fungi.[29, 30] Synergistic effects of the major and minor constituents present in the essential oils should also be taken into consideration.

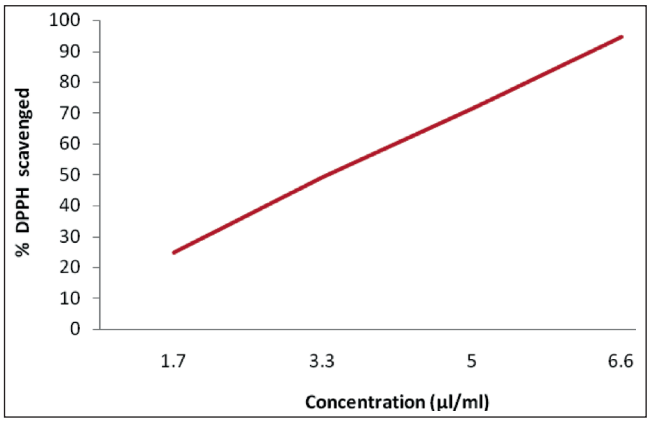

Figure 1: DPPH radical scavenging activity of the essential oil of the aerial part of *Plectranthus caninus*.

Free-radical scavenging activity

The antioxidant activity of the essential oil of *P. caninus* on DPPH was determined spectrophotometerically and the radical scavenging ability of the oil is shown in Figure 1. The oil reduced DPPH in a concentration dependent manner with an EC_{50} value of 2.89 mg/ml (3.5 µl/ml). The positive control, vitamin C, reduced DPPH with an EC_{50} value of 4.42 µg/ml.

The radical scavenging activity of the oil can possibly be due to the presence of α-pinene, γ-terpinene and terpinen-

4-ol which are reported in the literature to possess antioxidant activity.[31, 32]

Anti-inflammatory activity

The results of the anti-inflammatory test carried out on the essential oil of *P. caninus* showed that at doses of 200 and 300 mg/kg, the oil significantly lowered the increase in paw volume indicating possible anti-inflammatory activity of the plant (Figure 2, Table 3). Carrageenan-induced paw edema involves many mediators which induce inflammatory reaction in two different phases. The initial phase, which occurs between 0 and 2.5 h after the injection of the phlogistic agent, has been attributed to the action of mediators such as histamine, serotonin and bradykinin on vascular permeability. It has been reported that histamine and serotonin are mainly released during the first 1.5 h while bradykinin is released until 2.5 h after carrageenan injection. The oedema volume reaches its maximum approximately 3 h post treatment and then begins to decline. The late phase, which is also a complement-dependent reaction has been shown to be a result of overproduction of prostaglandins in tissues and may continue until 5 h post-carrageenan injection. [33]

According to the results of this study, the essential oil of *P. caninus* showed a dose dependant reduction of paw edema (Figure 2). The oil, at a dose of 200 and 300 mg/kg, was able to effectively inhibit increase in paw volume during the late phase (3 - 4 h after carrageenan injection) of inflammation and also exhibited inhibitory effect at early phase (Table 3). The effect of the oil at 300 mg/kg during the late phase of inflammation was even higher than that of indomethacin (10 mg/kg). However, no effect was observed at a dose of 100 mg/kg. Based on this observation and the biphasic nature of carrageenan-induced paw edema, it is possible to propose that the significant activity observed at doses of 200 and 300 mg/kg in the suppression of the late phase of inflammation may be due to the ability of the oil to inhibit prostaglandins.

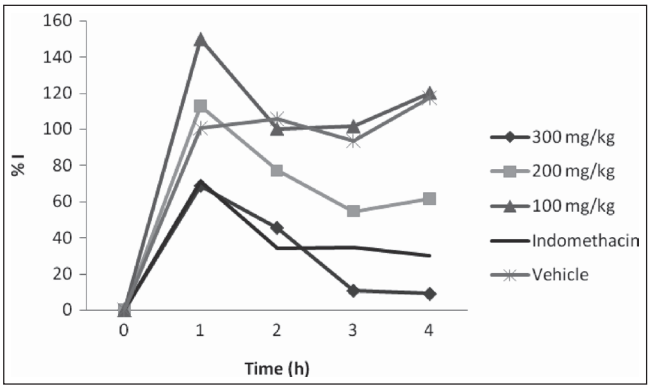

Figure 2: Comparison of the increase in paw volume of orally administered *Plectranthus caninus* essential oil at different doses with that of indomethacin (10 mg/kg). The activity of the oil at doses of 200 and 300 mg/ml is comparable to indomethacin ($p > 0.05$).

The anti-inflammatory activity of the oil may be attributed to the presence of camphor, α thujene, α-pinene, terpinen-4-ol and the other minor constituents with possible synergestic effects which have been reported in the literature.[34]

CONCLUSION

The essential oil of *P. caninus* is mainly composed of camphor and α-thujene. The oil possesses a broad-spectrum activity against bacteria and fungi and also significant free radical scavenging and anti-inflammatory activities. In view of the results of this study, the essential oil of *P. caninus* can be considered as a readily available natural antiinfective agent and preservative.

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Tabel 3: Inhibition of carrageenan-induced mouse paw oedema by the essential oil of *Plectranthus caninus*.

	1 h[a]	2 h[a]	3 h[a]	4 h[a]
Essential oil				
300 mg/ml*	38.96 ± 16.59	51.15 ± 17.91	91.07 ± 8.93	92.38 ± 3.60
200 mg/ml*	20.94 ± 29.55	32.42 ± 9.21	55.54 ± 24.73	65.10 ± 14.42
100 mg/ml	–120.64 ± 88.43	–11.81 ± 65.37	–27.80 ± 41.23	–38.36 ± 67.51
Indomethacin (10 mg/kg)	36.14 ± 11.37	66.23 ± 6.44	68.04 ± 15.41	73.61 ± 13.03

[a]Time after carrageenan injection
* Comparable activity to indomethacin ($p > 0.05$)

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Optimization Of Extraction Condition And Quantification Of Total Flavonoids In *Elaeagni Folium*

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ABSTRACT

Introduction: *Elaeagni Folium* has been used as a crude drug to cure lung deficiency cough, asthma and shortness of breath for a long time in China. The research aimed to optimize the extraction condition and measure the amount of total flavonoids in this medicinal material.

Methods: Reflux extraction was an efficient method, compared with sonication and soxhlet extraction, to extract total flavonoids. On the basis of single factor test, the $L_9(3^4)$ orthogonal test was used in the optimization of technological parameters by investigating the ethanol concentration, extraction time and the material /solvent ratios. Using 0.1 mol·L^{-1} aluminum chloride ($AlCl_3$) solution as chromogenic agent, the total flavonoids content was measured by ultraviolet and visible spectrophotometry.

Results: The optimal extraction parameters showed that: ethanol concentration was 85%, ratio of solvent to raw material 40:1 and extraction time 1.5 h. The amount of total flavonoids in the leaf of *Elaeagnus pungens* Thunb. collected in different harvest time and habitats was 20.8-33.2mg·g^{-1}.

Conclusions: It indicated that the amount of total flavonoids had significant difference in this crude drug from different habitats but at the same time, while the total flavonoids content was stable in the samples collected in different harvest time but at the same habitat.

Key words: *Elaeagni Folium.*; Total flavonoids; Extraction conditions; Orthogonal test

INTRODUCTION

Elaeagni Folium is the dried leaf of *Elaeagnus pungens* Thunb. subordinated to *Elaeagnaceae*. It is recorded by Compendium of Materia Medica that *Elaeagni Folium* has been used as a crude drug to cure lung deficiency cough, asthma and shortness of breath for a long time. The leaf is primary raw material for making "Hai Zhu Chuan Xi Ding Tablet", which treats bronchial asthma effectively [1-2]. Pharmacological experiments showed that the extract of *Elaeagni Folium* possessed obvious effects of preventing asthma, relieving cough and removing phlegm. It may conjecture that the effective fractions are flavonoids and alkaloids [3-4]. Researches also studied that the flavonoids isolated from the leaf such as kaempferol glucoside, kakkalide and 3'-O-methylquercetin significantly inhibited proliferation of human gastric carcinoma cell SGC -7901 and cervical cancer cells Hela in vitro[5] .Many studies also reported the chemical constituents of flavonoids were kaempferol, kaempferol glucoside, quercetin, 3'-O- methylquercetin and so on[6-7]. However, the extraction technique of total flavonoids in *Elaeagni Folium* has not been reported. Thus, the best condition of extraction was obtained by studying single-factor tests and orthogonal design test. With the yield of total flavonoids as an index, the amount of total flavonoids of *Elaeagni Folium* collected in different habitats and harvest time was measured. This study provided experimental foundation for quality control, exploitation and utilization of this crude drug.

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MATERIALS

Leaf of *E.pungens* Thunb. was collected in Hubei province of China and authenticated by Dr. Dingrong Wan, a professor in Pharmacy college of South-central University for Nationalities.

The standard rutin was purchased from National Institutes for Food and Drug Control of China (Batch

Table1: Comparation of flavonoids yield with different extraction method

method	Sample (g)	Volume of 70% ethanol (ml)	Extraction time (h)	Flavonoids yield (%)	Average (%)
Soxhlet extraction	4.0001	80	2	1.17	1.16
	4.0003	80	2	1.14	
Reflux extraction	3.9996	80	2	2.16	2.14
	4.0003	80	2	2.11	
Sonication	4.0001	80	2	1.92	1.95
	4.0002	80	2	1.97	

number: 100080-200707). Aluminum chloride crystal ($AlCl_3 \cdot 6H_2O$) and alcohol employed were analytical reagent.

METHODS AND RESULTS

Sample Preparation

The dried leaf of *E. pungens* Thunb. was pulverized. 1 g of this power was sonicated with 20 ml of 70% alcohol for 30 min .The testing solution was obtained by filtering the extraction.

Standard solution preparation

Weigh accurately 5.7mg rutin reference standard in 50 ml of volumetric flask, dissolve and make up to mark with 70% alcohol. A total of 0.114 mg·ml^{-1}of standard solution was prepared.

Detection wavelength selection

2 ml of rutin solution and 1 ml of the testing solution were accurately pipetted into a 25 ml volumetric flask respectively, with 3 ml of 0.1mol·ml^{-1} aluminium chloride($AlCl_3$) solution separately[8-10]. Then the mixture was diluted to volume with 70% alcohol and shaken up. After 30 min, the absorption spectra of testing solution and reference solution were gained by wavelength scanning at 300-600 nm, showing a maximum absorption wavelength at 410 nm.

Standard curve preparation

Each standard solution of rutin (0.00 ml, 1.00 ml, 2.00 ml, 4.00 ml, 6.00 ml, 8.00 ml) was spiked into 25 ml measuring flask and conducted by the method stated in "Detection wavelength selection". 30min later, the absorbance of the mixtures were measured at 410 nm using a spectrophotometer against an appropriate blank solution. Using the absorbability as ordinate and the concentration as abscissa, the specification curve was obtained. The calibration curve presented a linear response within the concentration range of 4.56-36.48 μg·ml^{-1}. The regression equation was $Y = 0.0233X + 0.0043$, $R^2 = 0.9997$.

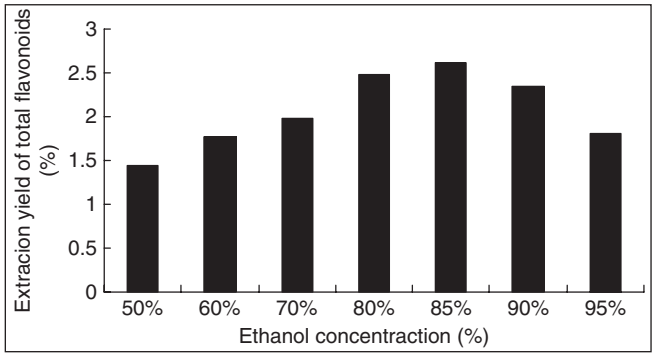

Figure1: Effect of ethanol concentration on the extraction yield of total flavonoids

Total flavonoids determination

1g of leaf was used in each extraction experiment with appropriate different concentration of alcohol.1 ml of each extract and 3 ml 0.1 mol·L^{-1} $AlCl_3$ were mixed in a 25 ml volumetric flask and the volume was made up with corresponding concentration of alcohol. The mixture was shaken and allowed to settle for 30 min at room temperature. The absorbance was measured at 410 nm. The extraction yield of flavonoids was calculated from the standard curve.

Optimization of extraction condition

Extract method selection To select efficient extract method, reflux, sonication and soxhlet extraction were compared. The results showed that reflux could provide the highest extraction yield (Table 1).

The single factor test

To determine the single factor experiment condition, the effect of three main factors including ethanol concentration, extraction time, material /solvent ratios were studied.

The extractions were performed with different concentrations of ethanol, namely 50%, 60%, 70%, 80%, 85%, 90%, 95% (v/v). Figure 1 showed that the total flavonoids amount increased with rise of concentration of ethanol from 50% to 85%. However, the flavonoids yield began to

decline, if the alcohol concentration continued to increase. Therefore, the optimal ethanol concentrations were 80%, 85% and 90%.

The effects of the material /solvent ratios from 1:10 to 1:40 on the extraction yield were investigated. Figure 2 manifested that the amount of flavonoids increased significantly with rise of the ratio (material/solvent) in a range of 1:10-1:30(w/v).Nevertheless, when the ratio was greater, the yield of extraction almost became steady. In the consideration of economy, the range of material / solvent ratios could be 1:20, 1:30, 1:40.

The effects of the extraction time from 1h to 2.5h on the extraction yield were measured. From figure 3, it could be found that the extraction yield improved with the increase of the extraction time from 1 to 2h, and didn't change significantly after 2h .Thus, the extraction time 1, 1.5, 2h were chosen for the following experiment.

Design for L9(34) orthogonal test

Considering the above experiment results, concentration of ethanol(A),material ratio(B) and extraction time (C) were selected as the experimental factors and a $L_9(3^4)$ orthogonal test was carried out. Factors and their levels were summarized in Table 2.

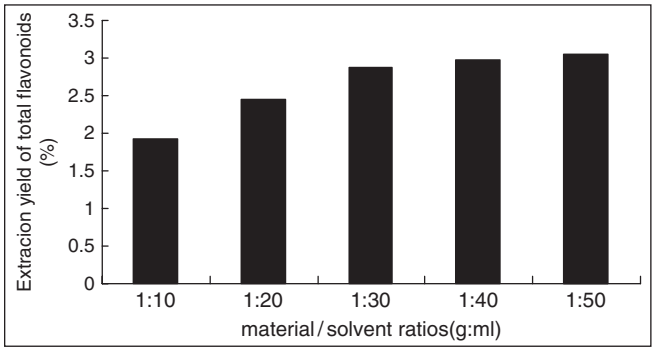

Figure 2: Effect of material ratios on the extraction yield of total flavonoids

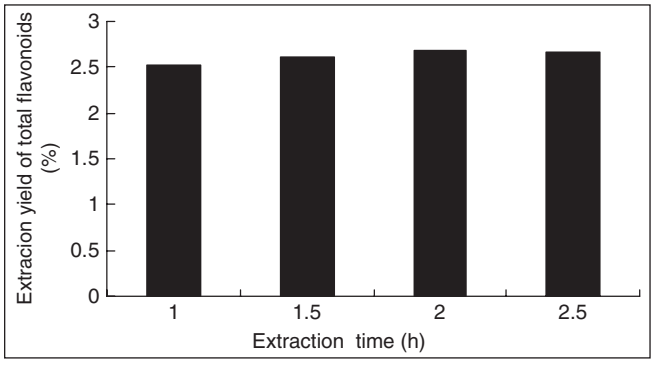

Figure 3: Effect of extraction time on the extraction yield of total flavonoids

The results of $L_9(3^4)$ orthogonal test were showed in Table 3, 4. Both results of intuitionistic analysis and variance analysis showed that the importance of three factors to affect extraction yield was B>A>C, with statistical significance. The optimal extraction condition was $B_3A_2C_2$, i.e., material/solvent ratios 1:40, concentration of ethanol 85% and extraction time 1.5h. Under the optimal conditions, the total flavonoids yield was 2.95%, which was higher than the orthogonal experimental result. This showed that orthogonal experiment was feasible.

Validation of the UV method

The repeatability was estimated by measuring the six samples under the optimal conditions in 1 day. The yields of total flavonoids were respectively 3.10%, 3.13%, 3.18%, 3.22%, 3.29% and 3.26%. The intermediate precision was evaluated by assaying the samples in duplicate on three consecutive days. The extraction yields were 3.15%, 3.11 % (day 1), 3.23%, 3.17% (day 2), 3.20%, 3.23% (day 3). Thus, the relative standard deviation (RSD) of repeatability and intermediate precision were 2.25% and 1.49% respectively. Recoveries ranged from 97% to 103 % with RSD2.25%, indicating the good accuracy of the proposed method.

Table 2: Factors and levels for orthogonal test

Level	Factors		
	A concentration of ethanol(%)	B ratios of liquid to solid	C extraction time(h)
1	80	20	1
2	85	30	1.5
3	90	40	2

Table3 : The results of $L_9(3^4)$ orthogonal experiment and range analysis

NO.	Factors				Flavonoids yield (%)
	A	B	C	D(Blank)	
1	1	1	1	1	2.20
2	1	2	2	2	2.70
3	1	3	3	3	2.54
4	2	1	2	3	2.45
5	2	2	3	1	2.56
6	2	3	1	2	2.87
7	3	1	3	2	1.67
8	3	2	1	3	2.44
9	3	3	2	1	2.63
k1	7.44	6.32	7.51	7.39	
k2	7.88	7.70	7.78	7.24	
k3	6.74	8.04	6.77	7.43	
k1'	2.48	2.11	2.50	2.46	
k2'	2.63	2.57	2.60	2.41	
k3'	2.25	2.68	2.26	2.48	
R	0.38	0.57	0.34	0.07	

Table 4: Variance analysis of the results

Source of error	Sum of Squares (SS)	Degree of freedom (f)	Mean square (S)	F value	P value	Significance
A	0.220	2	0.110	31.429	0.031	*(P<0.05)
B	0.553	2	0.276	79.000	0.012	*(P<0.05)
C	0.182	2	0.091	26.000	0.037	*(P<0.05)
D	0.007	2	0.004	1.000		

Table 5: Total flavonoids amount of samples collected in different harvest time and habitats

Harvest time	Habitat	Flavonoids amount (mg·g^{-1})	Average (mg·g^{-1})	RSD (%)
May 1, 2011	Huangmei, China	20.4		
		21.2	20.8	1.92
		20.8		
May 10, 2010	Wuhan, China	30.1		
		30.4	30.0	1.53
		29,5		
June 15, 2010	Wuhan, China	26.9		
		27.9	27.2	2.02
		27.0		
July 4, 2010	Wuhan, China	31.7		
		32.0	32.2	1.77
		32.8		
October 5, 2010	Wuhan, China	33.3		
		33.6	33.2	1.55
		32.6		

Sample determination

The samples collected in different harvest time and habitats were extracted by the optimal extraction method .According to the method illustrated in the "Total flavonoids determination", the total flavonoids content of each sample was also measured and the results were showed in Table 5.

DISSCUSION

To extract total flavonoids from *Elaeagni Folium*, the optimum condition obtained by using single-factor tests and orthogonal test was as follow: the ratio of solvent to raw material was 40:1, ethanol concentration 85% and extraction time 1.5 h. This result provided the reliable experimental basis for industrial production to extract the total flavonoids in this crude drug. Using rutin as standard substance and 0.1 mol·L^{-1} aluminum chloride ($AlCl_3$) solution as chromogenic agent, the total flavonoids content was measured by ultraviolet and visible spectrophotometry. The confirmation test indicated the optimal method was reproducible, accurate and feasible. Under this condition, the results represented that the amount of total flavonoids in the samples were 27.2-33.2mg·g^{-1} collected at Wuhan, Hubei Province but in the different harvest time from early summer to autumn. However, the total flavonoids content in this drug was 20.8mg·g^{-1} harvested in May at Huangmei, Hubei Province, which was tremendously different from the amount of the former. With total flavonoids as active ingredient, it indicated that the amount of total flavonoids was stable in this crude drug collected in different harvest time but at the same habitat, which provided basis for the further development and utilization.

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Phytochemical analysis and antioxidant activity of *Alstonia scholaris*

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ABSTRACT

The present study investigates the phytochemical constitution and antioxidant activity of various solvent (aqueous, butanol and ethyl acetate) extracts of the leaf and bark of *Alstonia scholaris*. The antioxidant assays performed include DPPH, ABTS and FRAP. The phytochemical screening revealed the presence of tannins, proteins, phenols and steroids in aqueous extracts of both leaf and bark of the plant. Butanol extract also showed moderate presence of the phytochemicals in the preliminary screening. The aqueous and butanol extracts showed significant antioxidant activity with DPPH, ABTS and FRAP assays when compared to the ethyl acetate extract. IC50 value of aqueous extract of bark and leaf were found to be 1.21 mg/mL and 2.83 mg/mL respectively in the DPPH assay and 210 µg/mL and 523 µg/mL in the ABTS assay. At 2 mg/mL concentration, the aqueous extract of bark and leaf showed ferric reducing power of 130.70 µmoles/mg and 109.52 µmoles/mg respectively in the FRAP assay. The TLC analysis of the aqueous extract of the bark, which showed highest antioxidant activity among the other extracts, was also performed.

Key Words: *Alstonia scholaris*, bark, anti oxidant activity, TLC

INTRODUCTION

Plants have been the basis of traditional medicines from time immemorial throughout the world and continue to provide new targets for remedies for many afflictions of mankind. The past couple of decades have seen considerable change in opinion regarding ethnopharmacological therapeutic applications of phytochemicals. A great deal of effort therefore still focuses on identifying and using these phytochemicals as source of novel therapeutic molecules. Antioxidants are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischaemia, asthma, arthritis, inflammation, neuro-degeneration, Parkinson's disease, mongolism, ageing process and perhaps dementia.[1] Antioxidant based drugs or formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last three decades. This has attracted a great deal of research interest in natural antioxidants. The plant kingdom has been described as a reservoir of many novel biologically active molecules of medicinal value.[2] Recently there has been a surge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury. *Alstonia scholaris* belongs to the family apocyanaceae which consists of about 250 genera and 2000 species of tropical trees, shrubs and vines.[3] Almost all parts of this plant are used in medicine and the bark has antihelminthic and astringent properties. It has been used in treating chronic diarrhea, dysentery and abnormal bowel movements.[4] The objective of the present study was to screen the phytochemicals and assess the antioxidant activity of the solvent extracts of bark and leaf of *Alstonia scholaris*. Free radical scavenging ability of the extracts were tested using antioxidant assays, viz., DPPH assay, ABTS assay and FRAP assay.

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MATERIALS AND METHODS

The bark and leaves of *Alstonia scholaris* were collected from VIT University campus, Vellore and authenticated by Plant Biotechnology division, VIT University, Vellore. The samples were washed thoroughly and dried. The dried samples were pulverized and 100g of the powered samples were refluxed with ethyl acetate, butanol and water in the ratio 1:10(w/v). The extracts were then concentrated using rotary flash

evaporator (Buchi type). The crude extract was used for the chromatographic profiling and different concentrations from the resultant crude extracts were used for assessing the antioxidant activity.

Phytochemical analysis

Chemical tests were carried out using standard procedures to identify constituents, as described by Harborne (1984), Trease and Evans (1979), and Sofowara (1993).[5-7]

Flavonoids: A few drops of NaOH solution was added to the extract solution (500 µL) followed by dil. HCl. The solution turned yellow and then colorless, indicating the presence of flavonoids.

Alkaloids: Presence of alkaloids was tested using 3 reagents namely, Dragendorff's reagent, Wagner's reagent and Mayer's reagent. To the extract solution (500 µL) a few drops of the reagent was added. A reddish brown precipitate indicated the presence of alkaloids.

Phenols and tannins: To the test solution (500 µL), a few drops of $FeCl_3$ were added. Presence of phenols and tannins was indicated by formation of a blue or blue-green colored solution (500 µL).

Proteins: A few drops of 4% NaOH were added to the extract (500 µL) followed by 1% $CuSO_4$. Violet or pink colored solution indicated the presence of proteins.

Carbohydrates: To the extract solution (500 µL), 1 to 2 mL of Anthrone reagent was added. Formation of green colored solution indicated the presence of carbohydrates.

Saponins: Few drops of Na_2HCO_3 were added to the extract solution (500 µL) and shaken for 5 minutes. Formation of froth or lather indicated the presence of saponins.

Glycosides: Few drops of aqueous NaOH were added to the extracts (500 µL). Yellow colored solution indicated presence of glycosides.

Steroids: Chloroform was added to the extract solution (500 µL) followed by conc. H_2SO_4 added slowly through the sides of the test tube. The lower sulphuric acid fraction turned brownish yellow and the upper layer turned reddish orange which indicated presence of steroids.

Antioxidant assays

DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay: The modified method of Blois (1984) was adopted for carrying out DPPH assay.[8,9] Ethyl acetate, butanol and aqueous extracts were tested for its DPPH radical scavenging activity. Different concentrations of the extracts (1, 2, 5, 10, 20, 30 and 50 mg/mL) were used for the assay. 1 mL of each extract was added to 1 mL of DPPH solution (1mM) and mixed thoroughly. The mixture was then incubated in the dark for 20 minutes at room temperature. After incubation the absorbance was measured at 517 nm.Control was a mixture of reagents alone without the extract. The decrease in absorbance was then converted to percentage antioxidant activity using the formula:

$$\% = 100 - \{[(Abs_{Sample} - Abs_{Blank}) \times 100] / Abs_{Control}\}$$

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) assay: To determine ABTS radical scavenging assay, a modified method of Re *et al.* (1999) was adopted.[10] The ABTS ((2, 2'-Azino-bis (3-ethylbenthiazoline-6-Sulphonic acid) reagent was prepared by mixing 2.28mg of Ammonium persulphate in 5 mL of water and 10mg of ABTS in 5 mL of water. After mixing the two solutions, the volume was made up to 50 mL with water and this solution was incubated for 6 hrs in dark at room temperature. Different concentrations of the extracts were prepared (0.1, 0.250, 0.5, 1, 2.5, 5 and 10 mg/mL). 500 µL of each of the extract solution was mixed with 1 mLof ABTS reagent. After mixing, the volume was made up to 1.1 mL with methanol. Absorbance was measured after 7 minutes at 734 nm. Blank used contained 500 µLof sample and the volume made up to 1.1 mL with methanol. Control used contained 1 mL ABTS reagent and the volume made up to 1.1 mL using methanol.

Percentage antioxidant activity was calculated using the formula:

$$\% = 100 - \{[(Abs_{Sample} - Abs_{Sample}) \times 100] / Abs_{Control}\}$$

FRAP (Ferric Reducing Ability of Plasma) assay: The determination of antioxidant activity using FRAP was performed using the modified method of Benzie and Strain (1996).[11] The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37 °C before use. The ethyl acetate, butanol and aqueous extracts taken for the assay were of 2mg/mL concentration. Each of the extracts (150 µL), were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark. Absorbance of the colored products (ferrous tripyridyltriazine complex) was measured at 593 nm. The results were expressed as µmoles/mg.

TLC: Thin layer chromatography was performed to generate a chromatographic profile of the aqueous

extract. 20 μL of the aqueous extract of bark was applied on the plate and developed to a distance of 8cm. The solvent systems, Toluene:Ethyl acetate:Formic acid (5:4:1) and n-Hexane:Chloroform (6:4) were selected after standardization.[5] After development, the plate was allowed to dry in air and sprayed with Anisaldehyde-Sulphuric acid reagent.[5]

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical screening results (Table 1 and Table 2) of the bark and leaf extracts in ethyl acetate, butanol and water showed the presence of all major phytochemical constituents excluding thiols. Tannins, proteins, phenols and steroids were found in higher proportions in the aqueous extracts of both bark and leaf when compared to other phytochemicals. The butanol extract showed presence of the bioactive phyto chemicals tested in lesser amounts when compared to the aqueous extract. Ethyl acetate drew out lower quantity of bioactive compounds from the plant sample compared to the other solvents. Khyade *et al.* reported the presence of alkaloids, saponins and tannins in the methanolic extract of the leaf of *A. scholaris*.[12] In a study by Thankamani *et al.*, it was reported that the solvent extracts (hexane, benzene, isopropanol, ethyl acetate, methanol and water) of flower of the plant showed the presence of alkaloids, which corroborates the fact that the plant is a rich source of alkaloids and can be used as a source of the same.[3] Amongst the chemical classes present in medicinal plant species, alkaloids stand as a class of major importance in developing new drugs because alkaloids own a great variety of chemical structures and have been identified as being responsible for the pharmacological properties of the medicinal plants.[13]

Table 1: Phytochemical analysis of extracts of bark of *Alstonia scholaris*.

Analytical Parameters	Water Extract	Ethyl acetate extract	Butanol extract
Flavonoids	+	+	+
Alkaloids			
Dragendroff reagent	+	–	–
Wagner's reagent	–	–	–
Mayer's reagent	–	–	–
Tannin	+++	+	++
Protein	+++	+	++
Saponin	+	+	+
Glycosides	+	+	+
Phenols	+++	+	++
Thiols	–	–	–
Steroids	+++	+	++
Carbohydrate	++	+	+

Table 2: Phytochemical analysis of extracts of leaf of *Alstonia scholaris*

Analytical Parameters	Water extract	Ethyl acetate extract	Butanol extract
Flavonoids	+	+	+
Alkaloids			
Dragendroff reagent	+	–	–
Wagner's reagent	–	–	–
Mayer's reagent	–	–	–
Tannin	+++	+	++
Protein	+++	+	++
Saponin	+	+	+
Glycosides	+	+	+
Phenols	+++	+	++
Thiols	–	–	–
Steroids	+++	+	++
Carbohydrate	++	+	+

Antioxidant assays

***DPPH assay*:** The results of the free radical scavenging potential of the different extracts tested by DPPH assay is given in the Table 3 and 4. Reduction of the DPPH radicals was observed by a decrease in absorbance where a change in the color to yellow denotes quenching of the free radicals by the plant extracts. Aqueous extract of the bark was found to have the most potent antioxidant property among all the other extracts with an IC50 value of 1.21 mg/mL

Table 3: DPPH radical scavenging by bark extracts of *Alstonia scholaris*.

	% of DPPH Scavenged		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
1 mg	49.41	−ve	24.95
2 mg	53.55	−ve	41.59
5 mg	67.94	−ve	44.84
10 mg	85.72	12.31	60.20
20 mg	92.39	17.90	61.19
30 mg	96.04	20.56	71.33
50 mg	>100	50.71	75.36

Table 4: DPPH radical scavenging by leaf extracts of *Alstonia scholaris*.

	% of DPPH Scavenged		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
2 mg	45.07	6.68	44.82
5 mg	62.74	12.05	81.39
10 mg	85.83	33.85	94.72
20 mg	89.15	78.60	97.69
30 mg	90.85	79.64	97.38
50 mg	91.62	80.69	91.95

followed by the aqueous extract of leaf with IC50 value 2.83 mg/mL. The high activity of water and butanol extracts are generally attributed to the presence of alkaloids and phenols, as the majority of active antioxidant compounds are observed in these classes of phytochemical compounds.

***ABTS assay*:** The aqueous and butanol extracts of the bark and leaf of *Alstonia scholaris* were found to be more effective scavengers of the ABTS radical compared to the ethyl acetate extract (Table 5 and 6). The IC50 values of aqueous extract of bark and leaf were found to be 210 µg/mL and 523 µg/mL respectively. The butanol and ethyl acetate extracts of the bark and leaf showed lower ability in scavenging the ABTS radical. Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals.[14]

Table 5: ABTS radical scavenging activity of bark extracts of *Alstonia scholaris*.

	% of ABTS Scavenged		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
100 µg	27.47	-	-
250 µg	57.76	10.95	29.01
0.5 mg	78.11	11.13	60.19
1 mg	86.37	13.60	79.09
2.5 mg	86.74	32.21	75.84
5 mg	85.64	61.16	78.73
10 mg	>100	69.71	84.67

Table 6: ABTS radical scavenging activity of leaf extracts of *Alstonia scholaris*.

	% of ABTS Scavenged		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
250 µg	13.36	−ve	4.98
0.5mg	47.67	11.57	28.90
1mg	79.74	53.90	45.89
2.5mg	78.87	57.98	63.95
5mg	85.60	16.29	64.20
12.5mg	88.03	9.91	67.01

***FRAP assay*:** The reducing ability of the extracts are tabulated in Table 7 and 8. At 2mg concentration, the aqueous extract of the bark showed maximum activity (130.70 µmoles/mg) followed by the aqueous extract of the leaf (109.52 µmoles/mg). The butanol extracts of bark and leaf showed lower activity than the aqueous extract, 108.28 µmoles/mg and 107.61 µmoles/mg respectively. The scavenging of the ABTS radical by the solvent extracts of *Alstonia scholaris* was found to be much higher than that of DPPH radical. Factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals.[15] Although many methods are available to determine antioxidant activity, it is important to employ a consistent and rapid method. While each method has its own merits and drawbacks, it has been found that the most common and reliable methods are the ABTS and DPPH methods; these have been modified and improved in recent years.[16] Ravi Shankar *et al.* (2008) had reported the antioxidant and free radical scavenging activity of the flower and fruit extracts of *A. scholaris*.[17]

***TLC*:** Thin layer chromatography was used to separate the individual chemical constituents of the aqueous extract of the bark of the plant. Toluene: Ethyl acetate: Formic acid (5:4:1) and n-Hexane: Chloroform (6:4) were used as solvent systems which showed good resolution. 11 bands (4 major and 7 minor) were visualized TLC was performed using Toluene: Ethyl acetate: Formic acid as the mobile phase. The bands became prominently visible after the plate was heated at105°C for 10 minutes. Pink and light blue colored bands were observed in both the solvent systems. 10 bands (2 major and 8 minor) were observed when n-Hexane: Chloroform (6:4) was used as the solvent system, indicating the presence of indole group of alkaloid compounds.[5] This TLC chromatogram can be used as a reference standard for further separation and isolation of compounds from the plant (Figures 1, 2 and 3). Analysis of phytochemical constituents of the species has been reported by many authors. Alkaloids have been found to be one of the major

Table 7: FRAP activity of bark extracts of *Alstonia scholaris*.

	(FRAP) mmoles AAE equivalents /mg tissue		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
2 mg	130.70 µmoles/mg	93.02 µmoles/mg	108.28 µmoles/mg

Table 8: FRAP activity of leaf extracts of *Alstonia scholaris*.

	(FRAP) mmoles AAE equivalents /mg tissue		
Sample Concentration	Water extract	Ethyl acetate extract	Butanol extract
2 mg	109.52 µmoles/mg	84.62 µmoles/mg	107.61 µmoles/mg

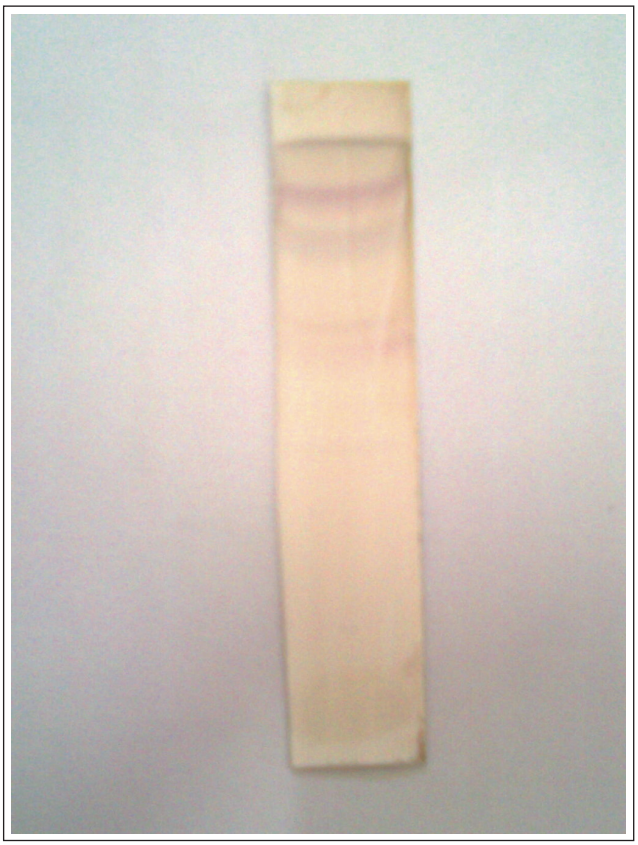

Figure 1: TLC of bark extract (T:EA: Formic acid)-11 Bands

Figure 2: TLC of bark extract – 11 bands on prolonged heating at 105°C

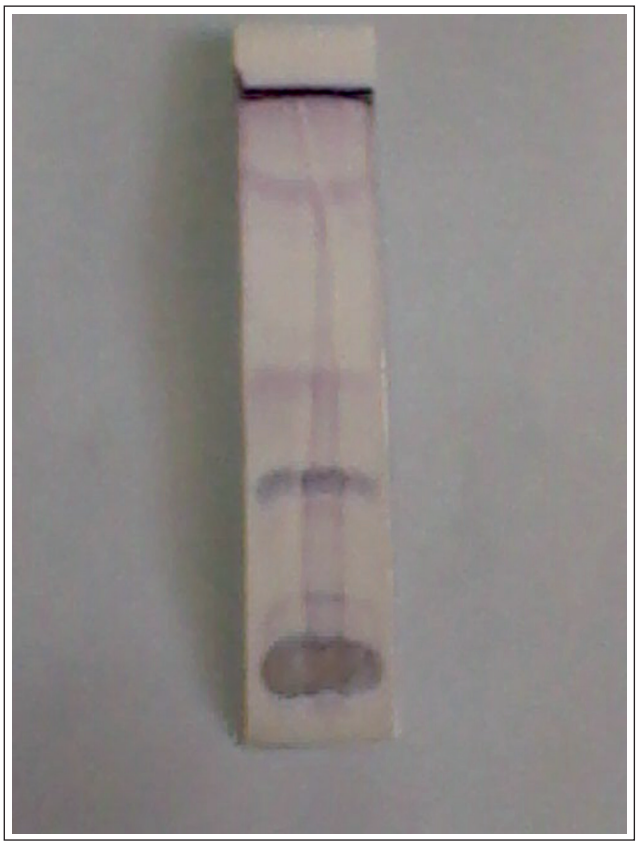

Figure 3: TLC of bark extract (n-Hexane: Chlroroform) - 10 bands indicating the presence of alkaloids

constituents of the species. Phytochemicals have been reported from different parts of the plant flowers, fruits, bark and leaves. Among the different alkaloids, Echitamine, Echitamine chloride Rhazine, monoterpenoid indole alkaloids and many other alkaloid derivatives have been reported.[18]

CONCLUSION

Phytochemical screening of three solvent extracts viz., Ethyl acetate, Butanol and Water of both bark and leaf revealed alkaloids, tannins, steroids and proteins as the major phytochemical constituents in various solvent extracts of *Alstonia scholaris*. The crude extracts were used for testing their antioxidant activity using DPPH, ABTS and FRAP assays. The aqueous extract was used for preparing a chromatographic profile using TLC. Aqueous extract of the bark was found to exhibit maximum antioxidant activity among all the extracts and the chromatographic separation of the extract into individual bands (mainly indole group alkaloids) showed the possibility for isolation and characterization of specific molecules from the crude extract, which could be purified and used for further studies. The ability of the extracts to scavenge different free radicals in different systems indicated that

they may be useful therapeutic agents for treating radical-related pathological damage.

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Development of Quality Standards of *Terminalia catappa* Leaves

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ABSTRACT

Sophisticated modern research tools for evaluation of the plant drugs are available today but microscopic method is one of the simplest and cheapest methods to start with for establishing the correct identity of the source materials. The leaves of *Terminalia catappa* (Combretaceae) are reported to have good medicinal values in traditional system of medicines. But the pharmacognostic and Phytochemical standardization of the leaves were not validated till date. With the aim of drawing the pharmacopoeial standards for this species, the present study deals with pharmacognostical parameters for the leaves of *Terminalia catappa* which mainly consists of Macroscopical and microscopical characters, physio-chemical constants, quantitative microscopy parameters and Preliminary phytochemical screening. This information will be of used for further pharmacological and instrumental evaluation of the species and will assist in standardization for quality, purity and sample identification.

Key words: Pharmacopoeial standards, Quality Standards, Standardization, *Terminalia catappa*.

INTRODUCTION

The usage of herbs to treat a variety of different ailments is universal, and exists in every human culture on Earth. Despite this, the largest use of medical herbs still occurs in societies which are not fully industrialized. Because of the high costs involved with manufacturing modern medicines, many people living in developing nations simply do not have the financial resources to pay for them, and as a result, they use natural herbs as an affordable alternative. There are a number of herbal systems that dominate the world today, and these systems are Ayurvedic medicine, Chinese herbs, Roman and Greek herbs and Shamanic herbs. The WHO has indicated that as many as 80% of all people living in the world make use of herbal medicine as their main source of healthcare [5]. It is no wonder that the world's one-fourth population i.e. 1.42 billion people, are dependent on traditional medicines for the treatment of various ailments [8]. Herbal formulations involve use of fresh or dried plant parts. Correct knowledge of such crude drugs is very important aspect in preparation, safety and efficacy of the herbal product [12] .However a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation and stringent quality control. There is a need for documentation of research work carried out on traditional medicines. With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as a medicine. The process of standardization can be achieved by stepwise pharmacognostic and Phytochemical studies. These studies help in identification and standardization of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy [16] .The leaves of *Terminalia catappa belonging to family* Combretaceae reported to posse's wide range of ethanomedical applications. In Suriname, a tea made from the leaves is prescribed against dysentery and diarrhea. It is also thought that the leaves contain agents for prevention of cancers and antioxidant as well as anticlastogenic characteristics and in Taiwan fallen leaves are used as a herb to treat liver diseases[4]. But the pharmacopoeial standards of the leaves were not produced till date.The current article describe some pharmacognostical, physicochemical and Phytochemical characteristics studied. The main objective of this study is to supplement some information with regards to its identification, Characterization and standardization of leaves of *Terminalia catappa* .

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MATERIALS AND METHODS

Collection of sample

Terminalia catappa was collected from local area in Pune. Their identity and Authentication was confirmed by Department of Pharmacognosy Marathwada Mitra Mandal's College of Pharmacy Pune by correlating their morphological and microscopical characters with those given in literatures. The remaining leaf samples were dried in shade. Coarse powder (60 #) of dried leaves of plants was stored for their microscopical study and phytochemical investigations.

Macrmorphology

The organoleptic characters of the fresh leaves and dried leaf powder like colour, odour and taste and the macroscopic characters viz, size, shape, surface, venation, apex, margin, base, texture were evaluated as per standard WHO guidelines[2,10,17,18].

Cytomorphology

The transverse section of fresh leaves and through the lamina and the midrib were cleared, mounted in glycerine water and observed under Digital microscope (MOTIC-B1). Microscopy of dried leaf powder was studied for evaluation of various parts present in given drug powder, [6,7,9,,14] the detail cytomorphological characters were observed and reported.

Quantitative Analytical Microscopy

Quantitative microscopy of leaf sample was performed as per WHO guidelines to determine various leaf constants[9,10,11,15,18].

Physicochemical Evaluation

Analysis of Physicochemical Constants of the ingredient has been done to evaluate the quality and purity of the powder drug. In physical evaluation, ash value viz., total ash, acid insoluble ash, water soluble ash, were evaluated. The ash value indicates the presence of inorganic salts present in the drug.The water soluble and alcohol soluble extractive values were determined[9,13] .The information collected from these test was useful for standardization and obtaining the quality standards. Determination of these physicochemical constants were done as per procedures mentioned in accordance with WHO guidelines[1,9,18].

Preliminary Phytochemical Investigations

The qualitative chemical tests carried out for the identification of the nature of phyto-constituents present in the powered crude drug [3,9].

RESULT AND DISCUSSION

Macrmorphologcal Description

Tropical-Almond is a 30 to 55-foot-tall, deciduous tree. The leaves of the plant are arranged alternatively with prominent veins, midrib and oil glands. The organoleptic evaluation of the leaf and leaf powder revealed that the leaf powder is pale green in color, with characteristics cynogenetic odour and taste. The results of macromorphology were mentioned in Table 1.

Cytomorphological Description

Fig 1 reveals the transverse section of the leaf through the lamina and the midrib region which shows the presence of dorsiventral type of cellular arrangement with a thick prominent midrib and thin lamina, the upper epidermis and lower epidermis was a made up of single layer of compactly arrange thin cell walled rectangular parenchyma cells. Mesophyll shows the cuboidal compactly arrange upper palisade parenchyma layer which was restricted and shows the presence of thick walled collenchymas cells and these are also present in a layer pattern above the lower epidermis, between these two layers a loosely arranged spongy parenchyma was observed. The midrib shows the vascular bundles arrangement which was similar to bicollateral type, major part of the vascular bundle was made up of lignified cell wall. The unicellular covering type epidermal trichomes were observed throughout the lamina and surface while the upper leaf surface shows the presence of paracytic epidermal stomata. Fig 2 shows the presence of upper epidermis and Mesophyll. Fig 3 and Fig 4 shows the presence of epidermal paracytic type of stomata and epidermal covering type of trichomes

Table 1: Macrmorphologcal Description

Sr. No.	Characters	Observation
Organoleptic characters		
1.	Colour	Dark Green
2.	Odour	Characteristic
3.	Taste	Astringent
Quantitative Macromorphology		
4.	Size	15–25 cm long and 10–14 cm broad
5.	Blade length	8-12 inch
Macroscopical features		
7.	Shape	Obovate
8.	Type	Simple
9.	Texture	Leathery
10.	Apex	Round and blunt
11.	Margine	Entire
12.	Base	Narrowly subcordate
13.	Venation	Pinnate

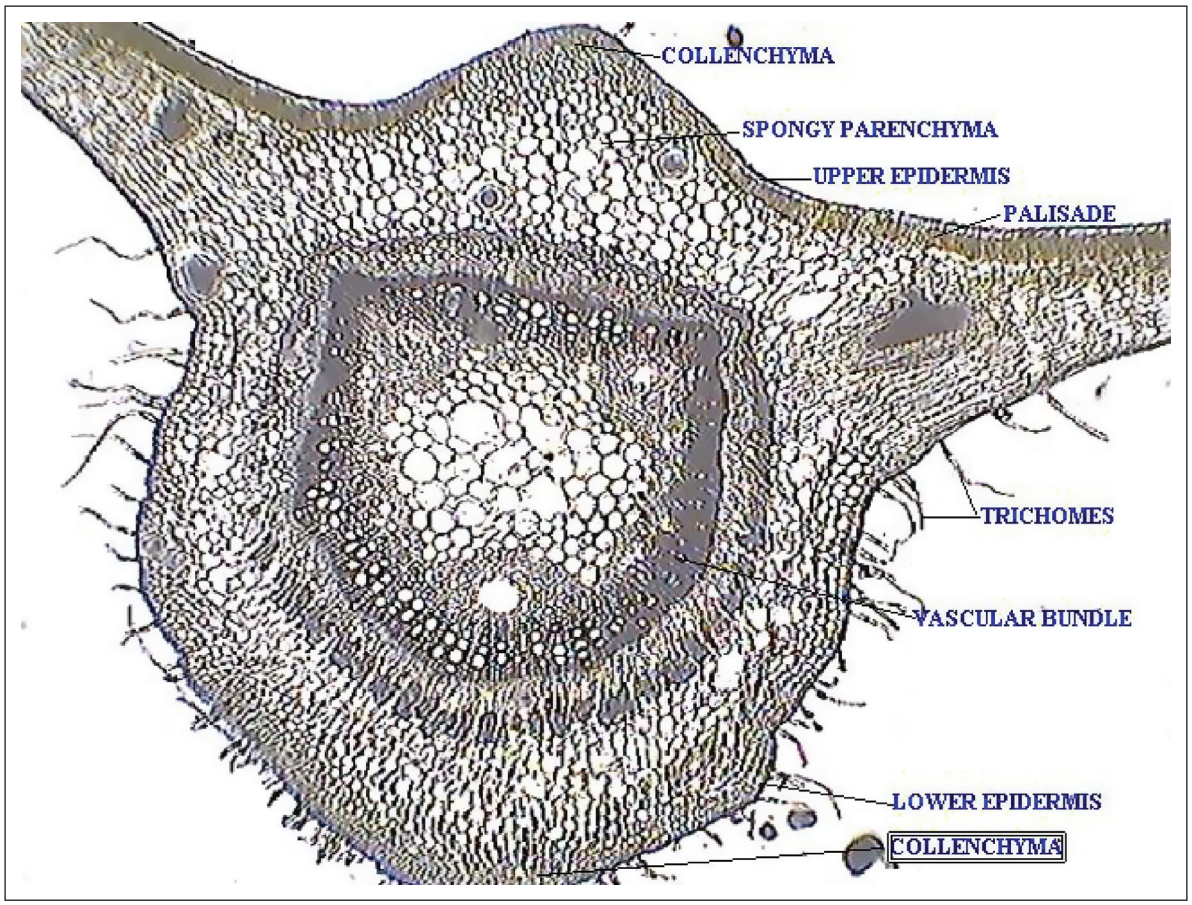

Figure 1: T.S oF *Terminalia catappa* Leaves

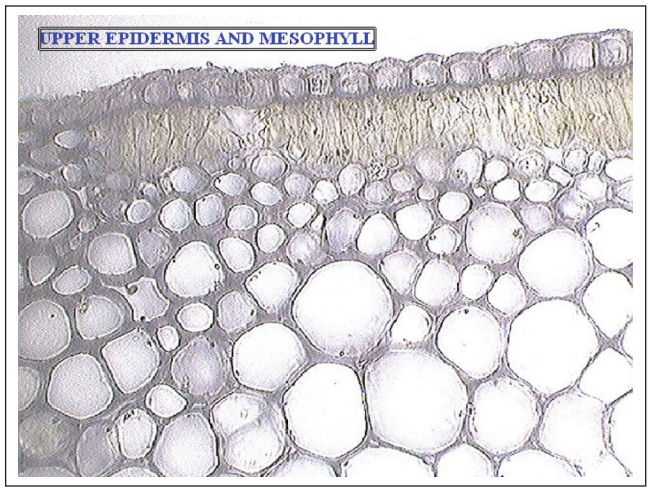

Figure 2: Upper epidermis and Mesophyll

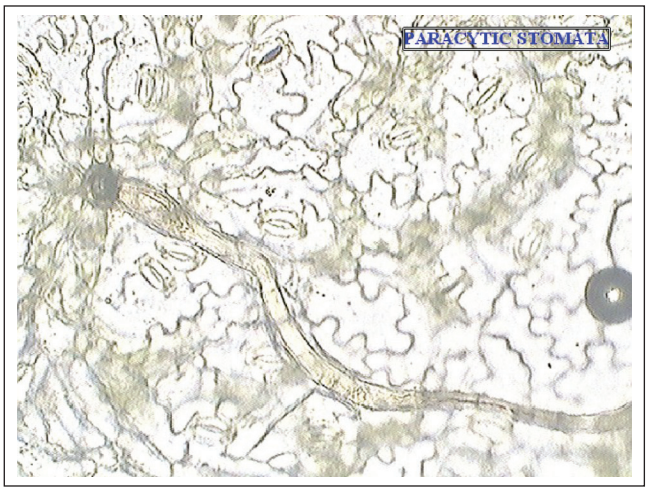

Figure 3: Epidermal stomata (Paracytic)

respectively. Fig 5 shows the presence of loosely arrange spongy parenchyma. Fig 6 reveals the presence of special type of bicollateral type of vascular bundle arrangement while fig 7 shows the presence of thick cellulosic walled collenchymas. In Powder microscopy fig 8 shows the presence of epidermal trichomes which are covering type, long thick unicellular and frequently curved near the base and fig 9 shows the epidermal stomatas are paracytic in nature with the two subsidiary cells parallel to the stomatal pore. Fig 10 shows Rectangular thin cell walled epidermal cells in surface view and the mesophyll appear to be in fragment manner showing dorsiventral arrangement.

Figure 4: Epidermal Trichomes (Covering)

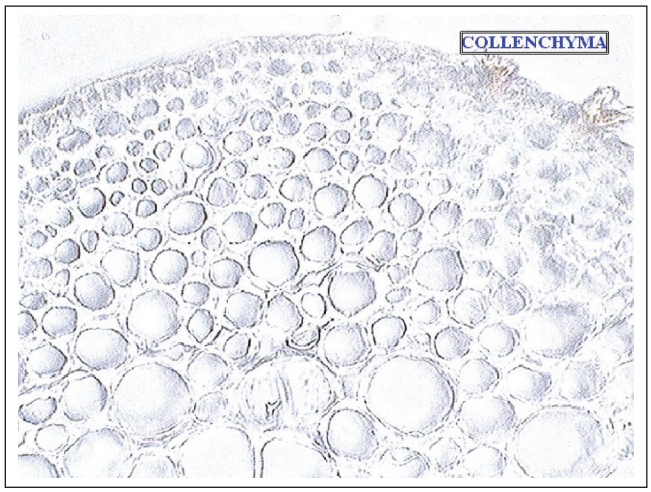

Figure 7: Collenchyma cell

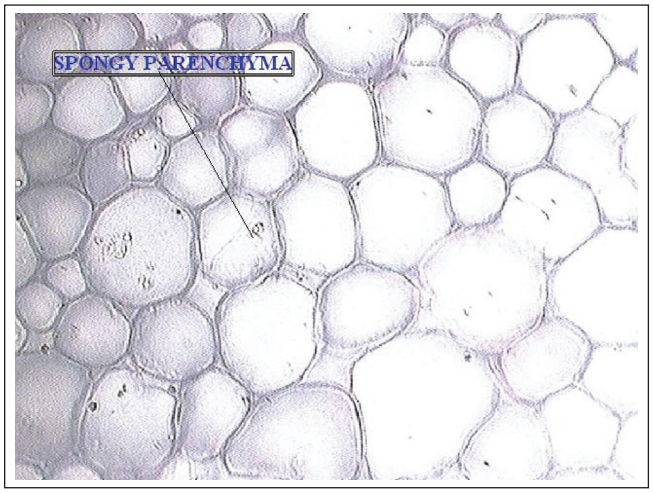

Figure 5: Spongy Parenchymav

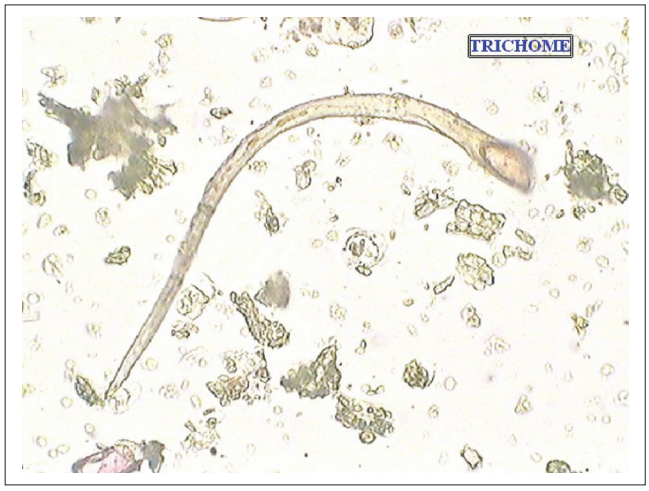

Figure 8: Powder Microscopy (Trichomes)

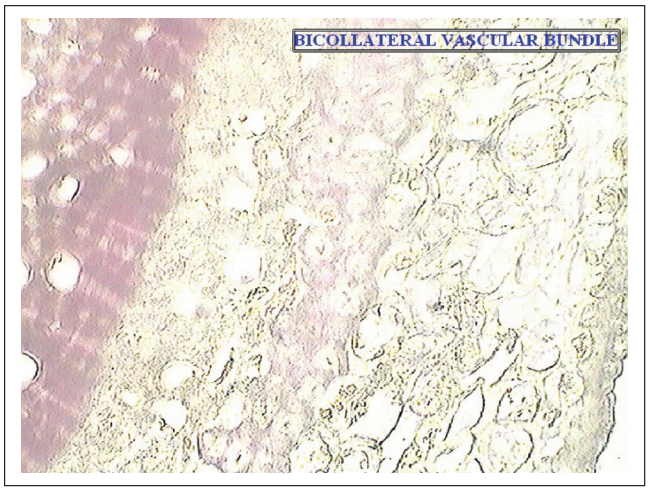

Figure 6: Vascular bundles

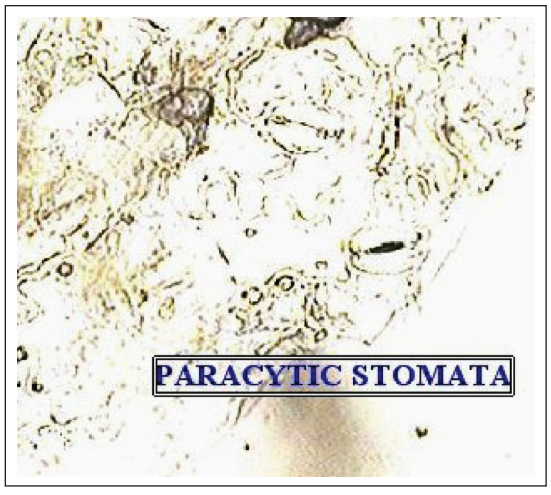

Figure 9: Powder Microscopy (Stomata)

Quantitative Analytical Microscopy

The method used for the measurement of cell contents of the crude drugs and thus helps in their identification, characterization and standardization. Such analysis can be applied to the positive identification of the botanical, geographical and other sources of a drug, and thereby, to evaluate the quality of drug. In addition to this, numerical data thus obtained can provide some information on physical properties of a drug and allow the estimation of the proportions of different cells in a drug. The physical dimensions of a sample obtained through this analysis are variable and depend largely on the exact treatment to which the material has been subjected. In any randomly selected sample, the cellular and subculture characters of some materials are constant, which will give a range of values. After a number of determinations, the characteristic feature/ number obtained is noted and compared to that within the range and standard deviation (SD)/ Standard Error Mean and we get the idea about the identity and characteristic feature of the drug. Results of the quantitative analytical microscopy were expressed in Table 2. As Vein islet is the term used to indicate the minute areas of the photosynthetic tissues encircled by the ultimate divisions of the vascular strands. A vein islet is the smallest unit of the tissue encircled by the ultimate divisions of the conducting strands of the leaves. Veinlet termination is an ultimate free end or termination of a veinlet. It can be used as a distinguishing character for the leaf of the same species or different one more particularly when the vein islet numbers does not give distinguishing results so the reproducibility of the result was much more useful here the results revealed the presence of thick mid rib portion for maximum absorption of water and the similarity of bicollateral type of arrangement confirm its nature, as the tree is present in tropical region which are confirmed by these value. The stomatal number and the stomatal index is a very specific criteria for identification and characterization of leafy crude drugs, the results of these are explain the maximum amount of the stomata present which signify the nature of tropical tree leaves. The results of the Quantitative Analytical Microscopy are expressed in Table 2.

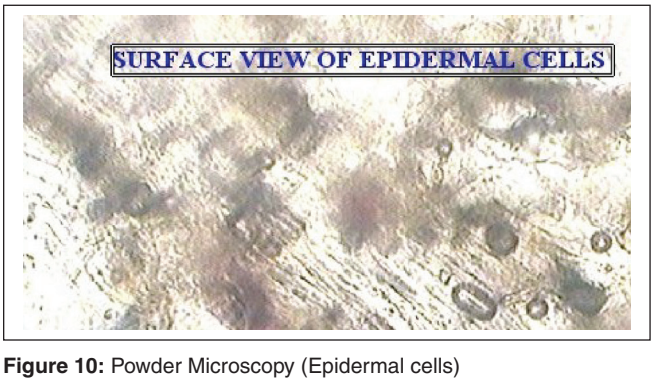

Figure 10: Powder Microscopy (Epidermal cells)

Table 2: Quantitative Analytical Microscopy

Sr. No.	Parameters	Value (In 1mm² area)
1.	Stomatal number (10x)	05.50
2.	Stomatal index (10x)	40.74
3.	Vein islet number (10x)	10.40
4.	Vein termination number (10x)	05.00

X = magnification power

Physicochemical Parameters

The results of the physicochemical constants of raw material lie within the limit which is mentioned in Table 3; this signifies that the quality and purity of raw material was good enough; the results of Ash values signify the content of inorganic material mainly the content of metallic salts and silica present in the raw material the values are 10.25 ± 0.62 % for total ash and 2.00 ± 1.08 % for acid soluble ash value; which are within fairly wide limit. Extractive values are used to determine the amount of active constituents in given amount of medicinal plants which is qualitative as well as quantitative estimation of phytoconstituents which act as a preliminary information about the drug; the water soluble extractive value found to be 28.33 ± 3.33% While the alcohol soluble extractive value was found to be 25.00 ± 2.88 % which signify the nature of the phytoconstituents present in plant. Insufficient drying favors spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles. Not only the ultimate dryness of the drug is important, equally important is the rate at which the moisture is removed and the condition under which it is removed thus the determination of moisture content also provide the method of preparation of drug; and it is observed that the moisture content of the drug was found to be 0.66 ± 0.16%w/w which signify that the drug is properly dried and properly stored. The swelling index gives an idea about the mucilage content which was found to be 0.13 ± 0.03 which was very less and negligible shows the absence of mucilaginous cells. As the pH was determined which was near to 5 which was in acidic range and may be because of acidic salts present in the leaves. The results of all Physicochemical parameters were expressed in Table 3.

Preliminary Phytochemical Screening

The preliminary phytochemical investigations of powdered leaves were performed which shows the presence of alkaloids, flavanoid type of major secondary metabolites

Table 3: Physicochemical Parameters

Sr. No.	Parameters	Standard
1.	Foreign organic matter (% w/w)	08.33±1.66
Ash Values		
2.	Total ash (% w/w)	10.25 ± 0.62
3.	Acid insoluble ash (% w/w)	02.00 ± 1.08
Extractive values		
4.	Alcohol soluble extractive value (% w/w)	25.00±2.88
5.	Water soluble extractive value (% w/w)	28.33±3.33
Physical Constants		
6.	Moisture content (LOD) (% w/w)	00.66±0.16
7.	P ^{H}10 % solution (% w/v)	5±0.00
8.	Swelling index (ml)	00.13±00.03

Table 4: Preliminary Phytochemical Screening

Sr. No.	Parameters	Observation
1.	Carbohydrates	+
2.	Amino acids	+
3.	Glycosides	+
4.	Flavonoids	+
5.	Volatile oil	+
6.	Alkaloids	+
7.	Tannins	+
8.	Steroids	+

+ indicates presence

which revealed their potent therapeutic activity. The results of the screening were express in Table 4.

CONCLUSION

Standardization is essential measure for quality, purity and sample identification. Macromorphology and Cytomorphology along with the Quantitative analytical microscopy is one of the simplest and cheapest methods to start with for establishing the correct identity of the source materials. Physiochemical and qualitative Chemical analysis of leaves confirm the quality and purity of plant and its identification. Here the information collected was useful for further pharmacological and therapeutical evaluation along with the standardization of plant material.

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Pharmacognostic Standardization and Phytochemical screening of Leaves of *Amaranthus spinosus* L.

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ABSTRACT

To ensure reproducible quality of herbal products, proper control of starting material is important. The first step towards ensuring quality of starting material is authentication. Thus, in recent years there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance. Despite the modern techniques, identification of plant drugs by pharmacognostic studies is more reliable. Pharmacognostical parameters of the leaves were studied with the aim of drawing the pharmacopoeial standards for this species. Macroscopical and microscopical characters, physico-chemical constants, phytochemical study for the presence of various secondary metabolites and HPTLC fingerprint profile of methanolic extract were studied. The present investigation on *Amaranthus spinosus* leaf might be useful to supplement information in regard to its identification parameters. Such studies are important in the way of acceptability of herbal drugs in present scenario of lacking regulatory laws to control quality of herbal drugs.

Key words: *Amaranthus spinosus*, HPTLC, Microscopy, Phytochemical, Standardization.

INTRODUCTION

Amaranthus spinosus Linn. (Family: Amaranthaceae) is commonly known as "Kate Wali Chaulai (Kanatabhajii)" in "Hindi", also used as vegetable and cultivated throughout in India, Sri Lanka and many tropical countries. In Indian traditional system of medicine (Ayurveda) the plant is used as digestible, laxative, diuretic, stomachic, antipyretic, improves the appetite, biliousness, blood diseases, burning sensation, leprosy, bronchitis, piles and leucorrhoea.[1] Previous reports of this plant showed that its extract was used for its anti-inflammatory properties,[2] effect on hematology,[3] immunomodulatory activity,[4] anthelmintic properties,[5] antiandrogenic activity and effect on biochemical changes in epididymis.[6] Chemically, it contains 7-p-coumaroyl apigenin 4-O-beta-D-glucopyranoside, a new coumaroyl flavone glycoside called spinoside, xylofuranosyl uracil, beta-D-ribofuranosyl adenine, beta-sitosterol glucoside, hydroxycinnamates, quercetin and kaempferol glycosides, betalains; betaxanthin, betacyanin; amaranthine and isoamaranthine, gomphrenin, betanin, b-sitosterol, stigmasterol, linoleic acid, 0.15% rutin and beta-carotene.[7-8] The present investigation deals with the study of some pharmacognostical characteristic of the leaves as a whole and in their powdered form.

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MATERIAL AND METHODS

Collection and authentication of plant material

The plant material was collected from the National Botanical Research Institute Garden, Lucknow and identified by a Taxonomist Dr. AKS Rawat with identification number CIF-RB-2-126-1. The leaves were separated, dried, coarsely powdered passed through sieve no 40 and stored in a closed container for further use. All reagents used were of analytical grade.

Morphological and Microscopical analysis

The macroscopical characters (size, shape colour, odour, texture, margin, base, apex and petiole) of the leaves were observed. Powder was identified with routine reagents to study the lignified cells, trichomes, stomata, fibers etc. Quantitative microscopy like measurement of Stomatal number, Stomatal index, Vein islet number, Vein termination

number, and Palisade ratio were determined by methods prescribed by Ayurvedic Pharmacopoeia. [9]

Qualitative microscopy

The required samples of leaves of selected plant were cut and fixed in FAA (formalin-5ml+ Acetic acid 5ml+ 70% Ethyl alcohol-90ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol as per the schedule.[10] Infiltration of the specimens were carried out by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were casted into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10- 12 µm. Dew axing of the sections were done by customary procedure.[11] The sections were stained with Toluidine blue as per the method.[12] Since Toluidine blue is a polychromatic stain, the staining results were remarkably good. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the protein bodies etc. Where ever necessary sections were also obtained with safranin and fast green and IKI (for starch). Photographs of different magnification were taken with Nikon Labphot 2.

Determination of physicochemical parameters

The Moisture content, ash values, extractive values with various reagents and were determined as per the Indian Pharmacopoeia.[13] The fluorescence characters of the powder with various reagents were observed under visible light and UV light (254 & 366 nm) as per the standard procedure.[14-15]

Preliminary phytochemical screening

Preparation of extract

The freshly collected leaves of *A. spinosus* were first air dried and then dried in tray drier under control conditions and powdered. The powdered leaves (1000g) were macerated with petroleum ether to remove fatty substances; the marc was further exhaustively extracted with of 50% ethanol for 3 days (3 × 3L) by cold percolation method and centrifugation at 10,000 rev/min. The extract was separated by filtration and concentrated on rotavapour (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under reduced pressure and thus 100 g of solid residue (yield 10 % w/w) was obtained. The extract was subjected to following preliminary phytochemical screening for the identification of various active constituents.[16-17]

HPTLC fingerprint profile

High performance thin layer chromatography (HPTLC) can successfully be employed for finger printing of *A. spinosus*. HPTLC analysis was performed on pre-activated (100 °C) silica gel G 60 F_{254} HPTLC plates (Merck) as stationary phase and plates were eluted in solvent system toluene: ethyl acetate (7: 3). After development, the plates were dried and densitometrically scanned at 310nm (WinCats software, CAMAG, Switzerland).

Sample preparation

2 g of extracts of *A. spinosus,* was dissolve in 25ml of methanol separately and used for HPTLC analysis. 10 µl of the test solution was applied and the plates were then developed in Toluene: ethyl acetate (7:3) to a distance of 8 cm. After development, the plates were dried and densitometrically scanned at 310nm.

RESULTS

Qualitative Microscopy

Macroscopic characters

Macroscopic and sensory evaluation of the leaves revealed that it is an erect, monoecious herb, up to 100–130 cm tall, much branched; stem terete or obtusely angular, glabrous or slightly pubescent, green or variably suffused with purple. The leaves alternate and are simple without stipules; petiole is approximately as long as the leaf blade; The blade shape is ovate-lanceolate to rhomboid, 3.5–11 cm × 1–4.5 cm, acute and often slightly decurrent at base, obtuse, rounded or slightly retuse and often short mucronate at apex, entire, glabrous or slightly pubescent on veins when young. The leaves were found to have characteristic odour and bitter in taste. (Figure 1)

Microscopic characters

Leaf has thick midrib projecting both adaxially and abaxially. The epidermal layer of thin midrib consists of small, squarish, thick walled cells with prominent cuticle. The palisade layer of lamina extends up to the lateral part of the hump. Midrib is deeply hallowed on the adaxial side and prominently semi-circular on the abaxial side. The abaxial part of the midrib is undulating in outline. The ground tissues are homogeneous, paranchymatous, thin walled and compact. The vascular bundles are collateral with broad circular thick walled vessels and parenchymatous xylem elements. Phloem fibers have a thick abaxial sheath. The vessel elements are 30-40 µm in diameters. Calcium oxalate crystals are abundant in the midrib. Some of them

Figure 1: Young plant of *A. spinosus*

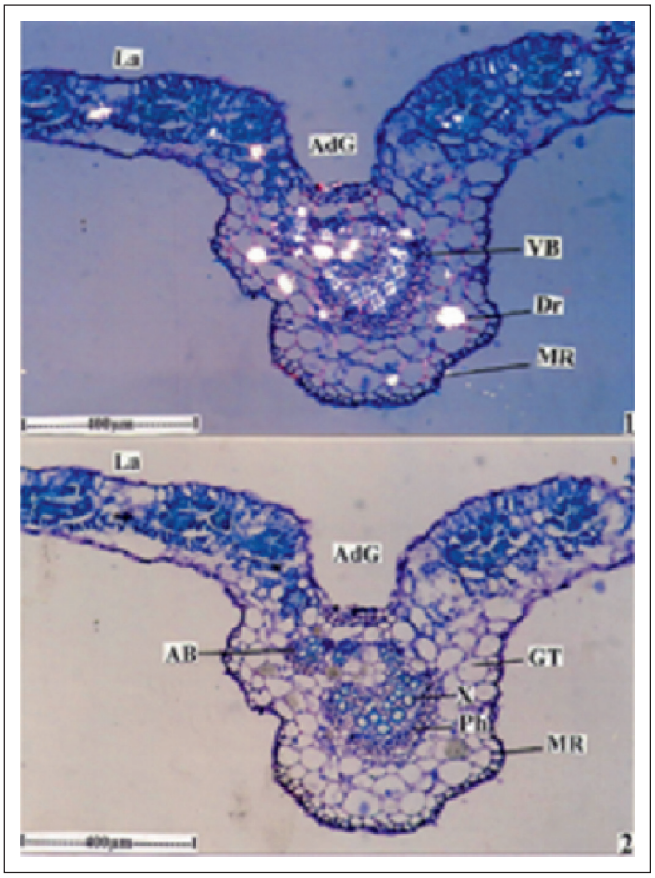

Figure 2: T.S. of leaf through midrib with lamina with crystals of calcium oxalate.
(Dr – Druses, VB – Vascular bundle, La – Lamina, Adg – Adaxial groove, AB – Accessory bundle, GT – Ground tissue, MR - Midrib, Ph – Phloem, X – xylem,)

are quite large measuring upto 50 µm in diameter and some of them are smaller measuring upto 20 µm in diameter. They occur in the ground parenchyma in the midrib and are also localized within the vessel wall. (Figure 2)

The lamina has wide radially oblong thick walled adaxial epidermis with prominent cuticle. The mesophyll tissue has narrow zone of adaxial palisade cells and three or four layers of small lobed spongy parenchyma cells. In middle part of the lamina, there are several circular small vascular bundles surrounded by bundle sheath cells called "Kranz-tissues (Figure 3)

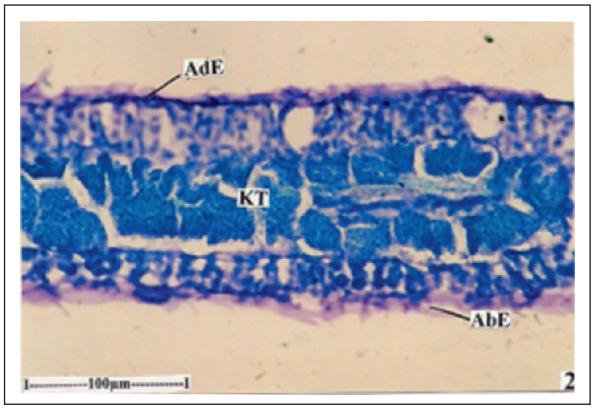

Figure 3: T.S. of Lamina
(AbE – Abaxial epidermis, AdE – Adaxial epidermis, KT – Kranz – tissue)

Powder Microscopy

Microscopic study of powder revealed the presence of fragments of abaxial epidermis with anomocytic stomata and multicellular, slightly lignified covering trichomes. Oval, rounded starch grains, pitted xylem vessels, which are present in bundles and also clusters type of calcium oxalate crystals also present. (Figure 4)

Quantitative microscopy

Quantitative microscopy of leaves of *A. spinosus* revealed the following data illustrated in Table 1.

Physicochemical parameters

Physicochemical parameters like foreign matter, percentage of moisture content, total ash, acid insoluble ash, water soluble ash, ethanol soluble extractive and water soluble extractive were determined and depicted in Table 2. The

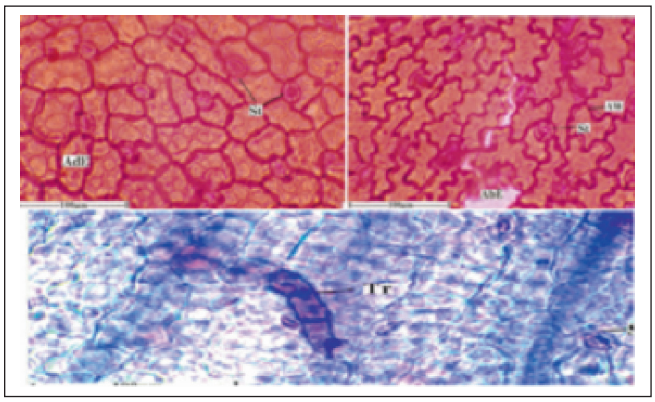

Figure 4: Powder Microscopy of *A. spinosus*
(AbE – Abaxial epidermis, AdE – Adaxial epidermis, Aw – Anticlinal Wall St – Stomata, Tr- Trichome)

Table 1: Leaf constants of *A. spinosus*

Leaf constants	Range	Average
Stomatal number (upper surface)	7-10	8.25
Stomatal number (lower surface)	107-122	112
Stomatal index (upper surface)	18.6-19.6	61.75
Stomatal index (lower surface)	33.2-41.0	36
Vein-islet number	35-38	36.25
Vein termination number	86-88	86.75
Palisade ratio (Apex : Middle : Base)	5.56 : 5.50 : 5.37	5.56 : 5.50 : 5.37

Table 2: Physico-chemical parameters of leaves of *Amaranthus spinosus* L.

Parameter	% w/w*	S.D.
Foreign matter	Nil	
Moisture content	20.2	0.56
Total ash	13.2	0.82
Water soluble ash	8.35	0.42
Acid insoluble ash	1.63	0.38
Sulphated ash	4.02	0.53
Alcohol soluble extractive value	4.825	0.42
Water soluble extractive value	10.125	0.49

*average of three readings

results of fluorescence analysis of the powder drug are mentioned in Table 3.

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of carbohydrate, phenolic compounds, phytosterols, alkaloids, flavonoids in 50% ethanolic extract of leaves. (Table 4)

HPTLC finger print profile

10 µl of the test solution was applicated on precoated HPTLC plate, the plate was then developed in the solvent system Toluene: Ethyl acetate (7: 3). The plate was then developed in the solvent system to a height of 8 cm, dried and scanned densitometrically at 310 nm, the peak area was recorded and the calibration curve was prepared by plotting the peak area against concentration of the component. The HPTLC profile of *A. spinosus* revealed the presence of nine spots at R_f 0.19 (3.09%), 0.29 (1.26%), 0.40 (8.01%), 0.47 (11.76%), 0.57 (10.72%), 0.63 (4.60%), 0.66 (1.92%), 0.75 (2.55%) and 0.93 (40.23%), The maximum percentage of area of component in the extract was found to be 40.23% w/w at Rf 0.93. (Figure 5)

DISCUSSION

The characters observed in the leaf like vein islet number, vein termination number, Stomatal number, Stomatal index, palisade ratio, covering trichomes cluster crystals of calcium oxalate of varying size, are some important diagnostic characters that are useful in determining the authenticity of the drug sample. The total percentage of ash values, acid insoluble ash, water soluble ash and percentage yield of extractives in different solvents, phytochemical screening and HPTLC are constant features of a part of the plant which may constitute individual drug. These reports would be of much significance in genuineness of the drug sample.

Table 3: Fluorescence analysis of *A. spinosus* powder

S.No.	Reagents	Color of the powdered drug	
		Day light	Ultraviolet light (254nm)
1.	Saturated picric acid	Pale yellow	Bright yellow
2.	50% Nitric acid	Light brown	Pale brown
3.	Hydrochloric acid	Light green	Brownish green
4.	Sulphuric acid (80%)	Dark brown	Light brown
5.	Glacial acetic acid	Light yellow	Colourless
6.	Iodine solution (N/20)	No change	No change
7.	Powder as such	No change	No change
8.	Sodium hydroxide (1N)	Pale yellow	Colourless
9.	Methanol	Dark Green	Green

Table 4: Preliminary phytochemical screening of the 50 % ethanolic extract of *A. spinosus*

S. No.	Constituents	Tests	50% Ethanolic extract
1.	Carbohydrate	Molish's test	+
		Fehling's test	–
2.	Fixed oil & fats	Spot test	–
		Saponification test	–
3.	Proteins & amino acids	Million's test	–
		Ninhydrin test	–
		Biuret test	–
4.	Saponins	Foam test	–
5.	Phenolic compounds	$FeCl_3$ test	+
		Pot. permagnate test	–
		Lead acetate test	+
6.	Phytosterol	Salkowiski test	+
		Libermann burchard test	+
7.	Alkaloids	Dragendroff's test	+
		Mayer's test	–
		Wagner's test	–
		Hager's test	+
8.	Gum & mucilage	Swelling test	–
9.	Flavonoids	Aqueous NaOH test	+
		Con. H_2SO_4 test	+
		Shinoda's test	–

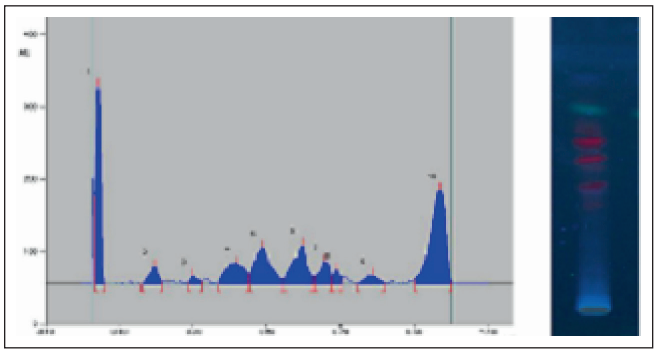

Figure 5: HPTLC fingerprint profile of *A. spinosus* in solvent system Toluene: Ethyl acetate (7:3) and scanned at 310nm.

In the present study, Pharmacognostical and phytochemical tests of leaves of *A. spinosus* were performed. The pharmacognostical studies comprises of taxonomic characters of the taxon, macro- and microscopical characters and diagnostic characters of the part used. The phytochemical parameters and physical constants were found useful in evaluating the pharmacopoeial standards. The qualitative phytochemical investigation gave valuable information about the different phytoconstituents present in the extract, which help the future investigators regarding the selection of the particular extract for further investigation of isolating the active principle.

CONCLUSION

The results presented in this study could serve as diagnostic parameters for proper identification as well as preparation of a monograph on *Amaranthus spinosus* Linn.

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Determination of Phyllanthin and Gallic Acid In Herbal Hepatoprotective Formulation By TLC-Densitometry Analysis

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ABSTRACT

Introduction: *Phyllanthus niruri, Andrographis paniculata and Embilica officinalis* are most important and high value medicinal plant known for its hepatoprotective activity. Several analytical methods including HPTLC and HPLC are reported for respective phytoconstituents. However, there is no simultaneous estimation of phyllanthin and gallic acid reported. The Objective of present work is to develop and validate HPTLC method for simultaneous determination of phyllanthin and gallic acid in polyherbal hepatoprotective formulation. **Method:** The method is employed TLC alumina plates precoated with silica gel 60F-254 as a stationary phase and toluene: ethyl acetate: formic acid (5:3.5:0.5 v/v/v) as a mobile phase. The standard markers namely phyllanthin and galic acid were identified in polyherbal formulation containing *P.niruri and E.officinalis* by R_f value. Densitometric analysis of phyllanthin and gallic acid was carried out at 254nm. **Results:** The precision of method was confirmed by relative standard deviation (RSD) which was lower than the 2%. This method was found to give proper separation of phyllanthin and gallic acid. The linear regression data for calibration plot shown good relationship with r^2=0.998 (phyllanthin) and 0.9991(Gallic acid) in the concentration range 5-9 µg/band. The method was validated for precision, accuracy, sensitivity, and specificity. **Conclusion:** statistical analysis proves that method is accurate and reproducible. The method is economical and can be employed for routine analysis of marketed polyherbal hepatoprotective formulation containing *P.niruri* and *E.officinalis.*

Key Words: Gallic acid, Hepatoprotective, TLC, Phyllanthin

INTRODUCTION

The genus *Phyllanthus* (Euphorbiaceae) are distributed in all tropical regions of the world from Africa to Asia, South America and the West Indies. *Phyllanthus niruri* is the most widely species found along roads and valleys, and on riverbanks and near lakes in tropical areas. Other species found in India are *P. fraternus*, *P. urinaria*, *P. virgatus*, *and P.maderaspatensis* and *P. debilis*. The genus *Phyllanthus* has a long history of use in the treatment of diabetes, intestinal parasites and liver, kidney and bladder problems. [1-2] *P. niruri* is highly valued in the treatment of liver ailments and has been shown to posses anti-hepatitis B virus surface antigen activity in both *in vivo* and *in vitro* studies. [3-4] Phyllanthin and hypophyllanthin are lignans, have been shown to be anti-hepatotoxic against carbon tetrachloride and galactosamine-induced hepatotoxicity in primary cultured rat hepatocytes.[5] The roots, leaves, fruits, milky juice and whole plants are used in medicinal preparations.

E.officinalis is reported to possess antioxidant, anticarcinogenic, antiulcer, hepatoprotective,, immunomodulatory activity.

Many herbal formulations, which are combinations of different herbal extracts, are used for the treatment of liver diseases. A large number of marked polyherbal formulations are reported for the hepatoprotective activity some of the formulations *viz* Livex, Liv-52, Hepatomed , Jigrin, Hepex, Jaudex, and Livol.[6-8] The most commonly herbs *viz Boerhaavia diffusa ,Eclipta alba ,Picrorrhiza kurroa, Oldenlandia corymbasa,Asteracantha longifolia ,Apium graveolens ,Cassia occidentalis,Cichorium intybus, Embelia ribes, Tinospora cordifolia, Andrographis paniculata* Nees, *Phyllanthus niruri*, *Phyllanthus emblica, and Trachyspermum ammi* are reported to have hepatoprotective activity. Amongst these plants *Andrographis paniculata (AP)* Nees, *Phyllanthus niruri (PN)* Linn and

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Phyllanthus emblica (PE) Linn are used for polyherbal hepatoprotective formulations.[9-11]

Thus, an appropriate analytical procedure for the quantitative determination of lignans in different *Phyllanthus* species is of considerable importance. Several analytical procedures involving HPLC[12-13] and HPTLC have been described.[14-16]

PN, PE and AP contain phyllanthin, gallic acid and andrographolide respectively as active constituent, is well known for its hepatoprotective activity from ancient times. Therefore, it was thought to develop herbal formulation containing these three drugs. This formulation was subjected to standardization by TLC-densitometric method using phyllanthin and gallic acid as marker compound.[17]

Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity[18]. TLC or High Performance Thin Layer Chromatography (HPTLC) is primarily used as an inexpensive method for separation, qualitative identification, or for the semi quantitative visual analysis of samples. TLC is thus often described as a pilot method for HPTLC However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields. The use of TLC/HPTLC have expanded considerably due to the development of forced flow (FF) and gradient TLC methods, stationary and mobile phase selection, as well as new quantitative methods.[19]

Several analytical methods are reported for estimation of phyllanthin from phyllanthus plant species. As there is no official HPTLC protocol for quantization of phyllanthin and gallic acid from polyherbal formulations therefore in the present paper we report an HPTLC method for quantitative analysis of phyllanthin and gallic acid from hepatoprotective Polyherbal formulation which provides good resolution of the peaks.This method will be utilize for routine analysis of marketed polyherbal hepatoprotective formulation containing *P.niruri* and *E.officinalis*.

MATERIAL AND METHODS

Instrumentation: A Camag HPTLC system (Muttenz, Switzerland) equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner 3, winCATS software and Hamilton (Reno, Nevada, USA) Syringe (100µL).

Material and reagents: HPLC grade alcohol, ethyl acetate, methanol, toluene, formic acid were obtained from S.D. Fine Chem Ltd (Mumbai, India).The biomarkers phyllanthin (Natural Remedies, Bangalore) and Gallic acid (Hi media, Mumbai) were used as working standards.

Preparation of standard stock solution

A stock solution containing 1mg ml^{-1} phyllanthin and gallic acid was prepared in methanol.

Preparation of sample for phyllanthin

Polyherbal tablet formulation was developed at laboratory scale. It contains a spray dried aqueous extract of *Phyllanthus niruri and E.officinalis*. Laboratory developed twenty tablets were weighed and crushed into fine powder. An accurately weighed 200 mg of powder was extracted with 50 ml alcohol on water bath; filtered through Whatmann filter paper no.42, evaporated on hot plate and residue was repeatedly washed (25x3) with hot water. The aqueous solution was successively extracted with (25x3) petroleum ether (60-80). The petroleum ether layer was discarded and aqueous layer was further extracted with ethyl acetate (25x3) in separator. Finally aqueous layer was discarded and ethylacetate solution was evaporated to dryness.[20] The Phyllanthin content was quantified by TLC-densitometric scanning.

Sample Preparation for Gallic acid

An amount of 200 mg of powder was extracted with 50 ml of methanol on water bath. Methanol evaporated under vacuum, then filtered through whatman paper No 42 and filtrate was evaporated to dryness.[24] The gallic acid content was analyzed by HPTLC.

Chromatographic conditions

Chromatograph was performed on 10x10 cm aluminum backed TLC plate coated with 0.2 mm layer of silica gel $60F_{254}$ ((E. Merck Ltd, Darmstadt, Germany) stored in a dessicator, application was done by Hamilton micorsyringe (Switzerland), mounted on a Linomat V applicator. Spotting was done on the TLC plate, ascending development of the plate, migration distance 80 mm (distance to the lower edge was 10 mm) was performed at 25 ± 2^0c with toluene: ethyl acetate: formic acid (5: 3.5: 0.5 v/v).as a mobile phase for phyllanthin and gallic acid, (Fig 1) in a camag chamber previously saturated for 30 min. samples were applied as 6 mm width at a spraying rate of 15s $\mu L^{-1.}$ The average development time was 15 minutes. After development the plate was dried at 50^0C in an oven for 5 minutes. Densitometric scanning was then performed with a Camag TLC Scanner 3 equipped with winCATS Software Version 1.3.0 at λ_{max} = 254 nm using Deuterium light source, the slit dimensions were 6.00 X 0.45 mm.

RESULTS

Validation of the method

The developed method was validated for following parameters;

Linearity

The stock solution (1mg/ml) of phyllanthin and gallic acid was prepared in methanol. Different concentration of the stock solution for phyllanthin and gallic acid (5-9 µg) were located to a plate to provide 5, 6,7,8,9 µg of phyllanthin and gallic acid/band separately. Each volume was applied three times on the TLC plate. Peak area data and the corresponding amounts were treated with linear least- square regression analysis. The calibration graphs were constructed by plotting peak area against amount of drug (µg spot $^{-1}$). The correlation coefficient, r^2, intercept and slope of phyllanthin was found to be 0.9998, 544.79, and 0.2145 respectively while for gallic acid was 0.9991, 32314.5 and 1.974. **Table 1**

Precision

The repeatability of sample application and measurement of peak area were expressed in terms of % R.S.D. and found to be 1.08 and 0.98 for phyllanthin and gallic acid respectively. The results depicted in Table 2 showed that no significant intra- and inter-day variation was observed in the analysis of phyllanthin at three different concentration levels. The % R.S.D. for intra- and inter-day analysis was found to be < 2% in all the cases. **Table 1**

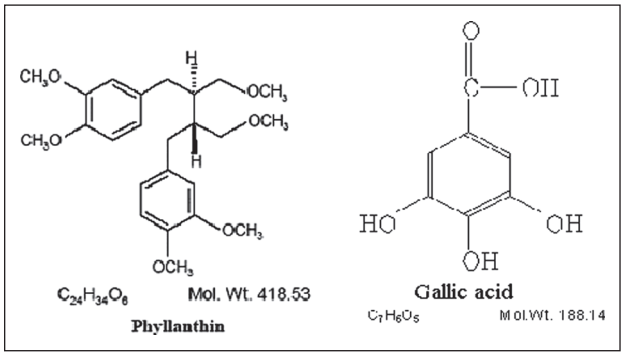

Figure 1: Structure of phyllanthin and gallic acid

Table 1: Summary of validation parameters

Parameters	Phyllanthin	Gallic acid
Linearity range (µg spot^{-1})	5 – 9	5-9
Correlation coefficient	0.998	0.9991
Slope	0.2145	1.974
Intercept	544.79	32314.54
Standard deviation	7.71	155.51
Precision (R.S.D. %)	1.08	0.98
LOQ &LOD (ng)	359.86 and 118.75	787.79 and 259.95
Robustness	Robust	Robust
Specificity	Specific	Specific

Sensitivity

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spot- ted six times. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of phyllanthin and gallic acid until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations. These were calculated by the use of the equations LOD= 3 X δ/s and LOQ= 10 X δ/s; Where, δ is the standard deviation of the peak area of the drugs (n=3) and s is the slope of the corresponding calibration plot. LOQ and LOD for phyllanthin were 359.86 and 118.75 ng, respectively. For gallic acid LOQ and LOD were found to be 787.79 and 259.95 ng respectively. **(Table 1).**

Accuracy (% recovery)

To ensure the accuracy of the proposed method, recovery studies for phyllanthin and gallic acid were performed by standard addition method at 80,100 and 120 % concentration levels of the phyllanthin. The mean percentage recovery for phyllanthin and gallic acid was found to be 98.21 and 97.19 respectively **(Table 3).**

Specificity

It was observed that the phytoconstituents of the other aqueous extracts of herbs present in the formulation and pharmaceutical excipients did not interfere with the peak of phyllanthin and gallic acid. Therefore, the method was specific. The overlap in the spectrum of standard phyllanthin and gallic acid sample extracted from formulation showed good correlation.

***UV Spectrum*:** The standard spot with the same R_f was located in the fingerprint of the formulation and confirmed

Table 2: Percentage amount of Phyllanthin and gallic acid present in Hepatoprotective formulation.

Sample	Amount found ng spot^{-1}		% w/w	
	Phyllanthin	gallic acid	Phyllanthin	gallic acid
Hepatoprotective Formulation	0.41	3.060	41.63	61.21

Table 3: Recovery studies for Phyllanthin and gallic acid

	Amount added %	Amount found (ng) mean ± S.D	% Recovery	Average recovery
Phyllanthin	80	5529.473±2.27	98.78	
	100	6839.523±3.89	97.73	98.21
	120	8242.177±2.00	98.12	
Gallic acid	80	5357.447±3.01	95.69	
	100	6879.657±1.56	98.28	97.19
	120	8200.423±2.31	97.62	

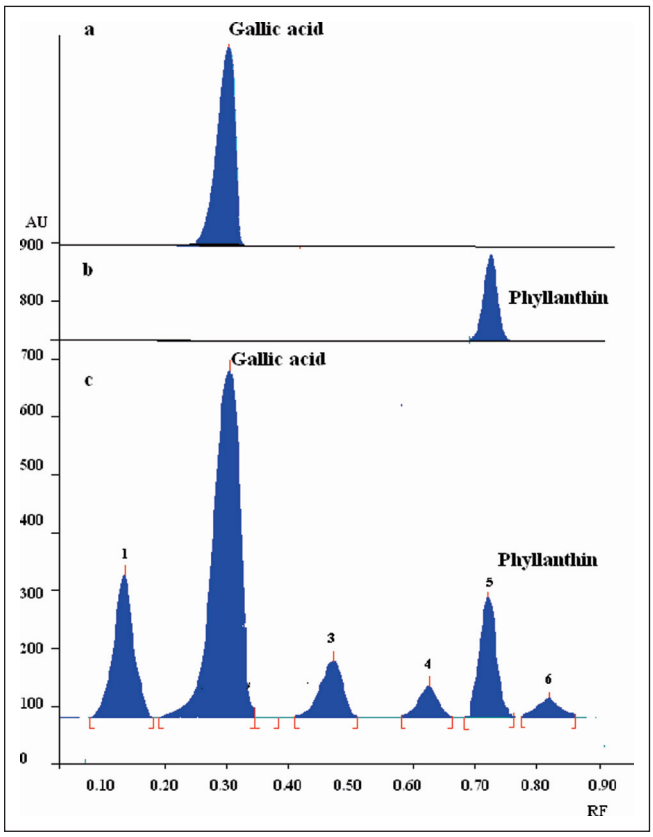

Figure 2: Chromatogram of standard gallic acid (a) (R_f: 0.30 ± 0.02); standard phyllanthin (b) (R_f: 0.72 ± 0.02); Polyherbal tablet formulation (c). Mobile phase: toluene: ethyl acetate: formic acid (2.5: 5.5: 0.5% v/v)

by superimposible spectra. At the shorter wavelength in the UV spectrum of the spots in standard and formulation are different but the shape is identical at longer wavelength. A peak purity spectrum of Phyllanthin and gallic acid was found to superimposible **(Figure 3.A & B).**

Analysis of the marketed formulation

Six spots at *Rf* 0.13, 0.30, 0.49, 0.63, 0.72 and 0.82 (Fig.2) were observed in the chromatogram of herbal drug samples extracted from tablet. There was no interference in analysis of phyllanthin and gallic acid from the other components present in the tablet formulation. These components appear in the chromatogram at significantly different *Rf* values. The total phyllanthin and gallic acid content was found to be 41.63 and % (w/w) in tablet formulation. Statistical evaluation of the results was performed with regard to accuracy and precision using Student's *t*-test and the *F*-ratio at 95% confidence level. The low % R.S.D. value indicated the suitability of this method for routine analysis of phyllanthin and gallic acid in pharmaceutical dosage form. **Table 2**

CONCLUSION

Herbal medicine is still used for primary healthcare because of better cultural acceptability, better compatibility with the human body and lesser side effects. So the multi-component herbal formulations can be standardized with newer techniques such as High performance thin layer chromatography. It emphasizes an integral formulation of pharmacologically active and phytopharmaceutically characteristic component of samples with similar and different attributes.[21] This is the most significant method which can be used for routine herbal drug analysis and for quality assurance. The proposed HPTLC method was found to be rapid, simple and accurate for quantitative estimation of phyllanthin and gallic acid present in formulation prepared at lab scale.The recovery values of phyllanthin and gallic acid was found to be about 98.21 and 97.19 shows the reliability and suitability of the method.

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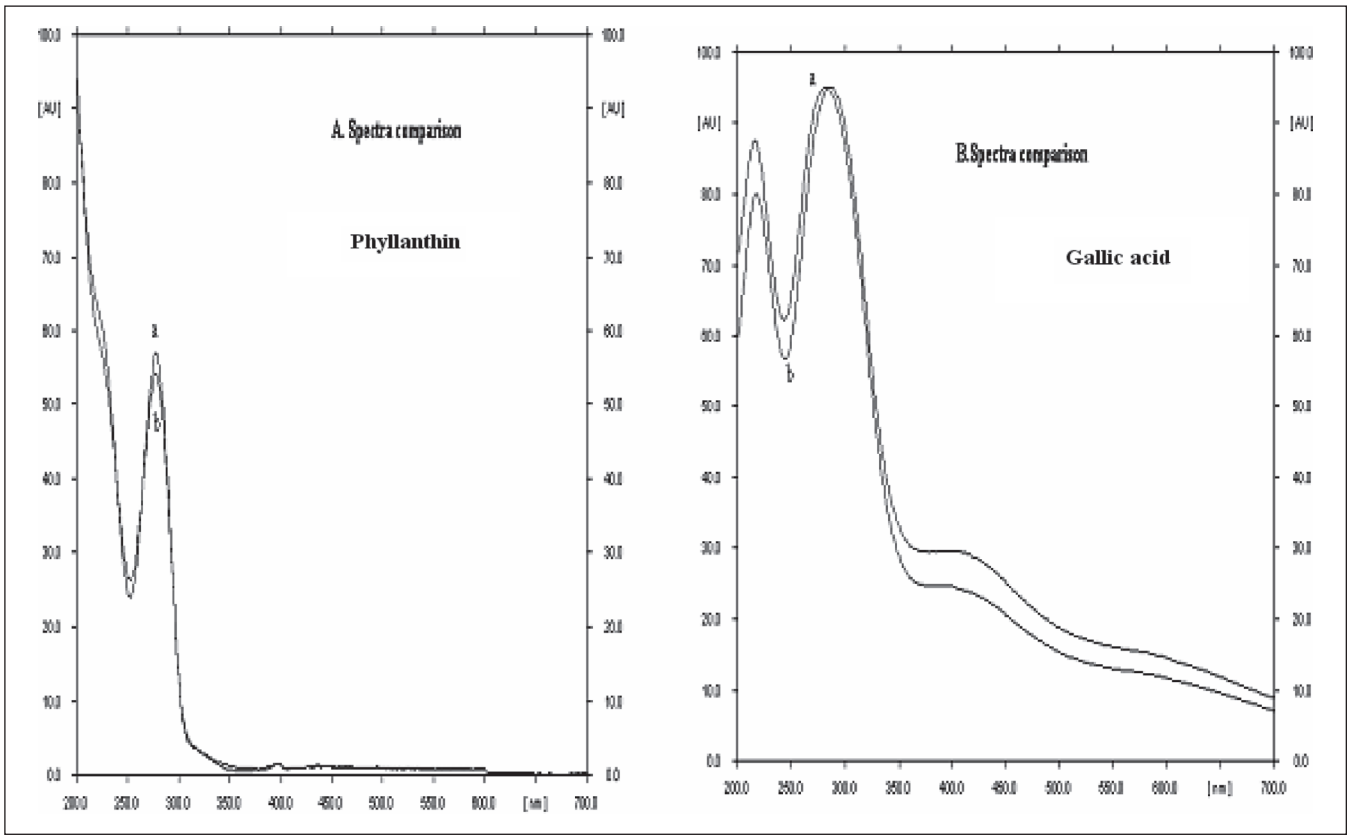

Figure 3: A: Peak purity spectrum of a) standard Phyllanthin (R_f 0.72) and b) Phyllanthin present in formulation (R_f 0.72). **B**: Peak purity spectrum of standard gallic acid (R_f 0.55) and gallic acid present in formulation (R_f 0.55).

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Development of Fingerprinting Methods of Balacaturbhadrika Churna: An Ayurvedic Formulation

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ABSTRACT

Balacaturbhadrika churna is an Ayurvedic formulation widely used in diarrhoea, fever, cough and asthma. The present article is an attempt to establish the scientific basis of one of the reputed Ayurvedic formulation. Investigations were carried out to study the physicochemical, phytochemical and spectrophotometric analysis of formulation. The values of percent loss on drying, angle of repose, Hausner ratio, Carr's index of the lab formulation were calculated as 6.84 ± 0.224, 27.36, 1.25 and 20 respectively, which indicates the moisture contents of formulation is within the range and depict good flow characteristics. Total ash, acid insoluble ash and water soluble ash were found 8.148 ± 0.337, 3.281 ± 0.286, and 45.602 ± 0.414 respectively; the value of total ash indicates the inorganic contents of formulation are below the standard limits, above stated results were also compared with marketed formulation. Alcoholic and aqueous extracts of formulations and ingredients were prepared and evaluated for phytochemical analysis and the results of extractive values shows higher alcoholic extractive value (39.294±2.226) of formulation depict that alcohol is a better solvent for extraction. Three laboratory batches of formulation and Piper longum powder were estimated for their piperine content against standard piperine solution on double beam UV-Visible spectrophotometer at λ max 342.5 nm.

Key words: Balacaturbhadrika churna, physicochemical properties, phytochemical properties, spectrophotometric analysis.

INTRODUCTION

In the past decade, there has been renewed attention and interest in the use of traditional medicine (Ayurveda, Yoga, Naturopathy, Unani, Siddha and Homeopathy) in India and globally. Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain [1]. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them [2-3]. The utilization of plants for medicinal purposes in India has long history, and the proportion of medicinal plants is the highest proportion of plants known for their medicinal purposes in any country of the world for the existing flora of the respective country. Medicinal plants are essential natural resource which constitutes one of the potential sources of new products and bioactive compounds for drug development [4].It is estimated that 80% of the population in rural India use medicinal plants to meet primary health care needs [5]. Continuous erosion in the traditional knowledge of many valuable plants for medicine in the past and the renewal interest currently, the need existed to review the valuable knowledge with the expectation of developing the medicinal plants sector [6]. Under the parasol of traditional medicine systems the Ayurvedic system of medicine also gaining global acceptance due to its amazing clinical efficiency. While Ayurvedic systems of medicines have long been used, there is negligible documented evidence regarding its safety and effectiveness. The lack of evaluation has, in turn, slowed down the development of regulations and legislation. Recently Good Manufacturing Practices (GMP) rules for Ayurvedic medicines to ensure the quality of the manufactured drugs and gain credibility to make them acceptable globally. The Drugs and Cosmetic Act 1940 controls the standards of manufacturing, sale and distribution of Ayurvedic drugs [7]. Balacaturbhadrika churna is a fine powder form, which is widely used in Diarrhoea, Fever, Cough and Asthma at dose of 500 mg to 1 gm/day [9]. It is composed of Ghana (musta), Krsna (pippali), Aruna (ativisa) and Sringi (karkatasringi). All the ingredients are firstly powdered separately and mixed together.

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In the present study physicochemical, phytochemical and spectrophotometric evaluation of the Ayurrvedic formulation Balacaturbhadrika churna and its ingredients has been carried out because these evaluations are amazingly uncharted till date and determination of these parameters are incredibly essential to assure the quality, safety and efficacy of this formulation.

MATERIALS AND METHODS

Materials

All the plant materials such as Ghana (*Cyperus rotundus*), Pippali (*Piper longum*), Ativisa (*Aconitum heterophy*) and Sringi (*Pistacia integerrima*) were purchased from the local market of Raipur, C.G. and identified morphologically and microscopically and compared with standard pharmacopoeial monograph. All the reagents and solvents used were of analytical grade. The ash values, extractive values with various reagents and were determined as per the WHO guidelines[9].

Preparation of formulation

Formulation was strictly prepared as prescribed in the official book of Ayurvedic Formulary of India (2003) [8]. 50 grams of each ingredients which includes Ghana (musta), Krsna (pippali), Aruna (ativisa) and Sringi (karkatasringi) were taken. All the ingredients were weighed accurately and made fine powder by passing through sieve no. 80. Fine powders were mixed geometrically in plastic tray and packed in plastic container.

Determination of ash values

Total ash

4gm of the each powdered material was accurately weighed and placed in a previously ignited and tared silica crucible. The material is spread in an even layer and ignited by gradually increasing the heat to a temperature of 500-600°C until it is white, indicating the absence of carbon. The material is Cooled in a desiccators and weighed. The content of total ash is calculated in mg per gm of air-dried material.

Acid-insoluble ash

To the crucible containing the total ash, 25 ml of hydrochloric acid is added, covered with a watch-glass and boiled gently for 5 minutes. The watch-glass is rinsed with 5 ml of hot water and this liquid is added to the crucible. The insoluble matter is collected on an ash less filter-paper and washed with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter is transferred to the original crucible, dried on a hot-plate and ignited to constant weight. The residue is allowed to cool in a suitable desiccator for 30 minutes, and then weighed without delay. The content of acid-insoluble ash is calculated in mg per gm of air-dried material.

Extractive values

The extractive values were recorded in alcohol and water with a view to study the distribution of various constituents of Balacaturbhadrika churna, and all raw ingredients of formulation. Accurately weighed 4.0g of coarsely powdered air-dried material was placed in a glass-stoppered conical flask and macerated with 100ml of the solvent for 6 hours, shaking frequently, and then allowed to stand for 18 hours. The mixture is filtered rapidly taking care not to lose any solvent. 25 ml of the filtrate is transferred to a tared flat-bottomed dish and evaporated to dryness on a water-bath. The residue is dried at 105°C for 6 hours, cooled in a desiccator for 30 minutes and weighed without delay.

Qualitative phytochemical studies

To detect the presence of various phytoconstituents in formulation as well as in raw materials phytochemical investigations were performed. The tests were performed on alcohol and water extracts. Qualitative phytochemical analyses were done for Balacaturbhadrika churna and all the raw ingredients of formulation [10-11]. Alkaloids, carbohydrates, glycosides, tannins and phenolic compounds, flavonoids, fixed oils, saponins, proteins and amino acids and steroids.

Bulk density

A sample of about 50 cm3 of each powdered ingredients that was previously passed through a U. S. Standard no. 20 sieve is carefully introduced into a 100 ml graduated cylinder. The cylinder is dropped at 2-sec intervals on a hard wooden surface three times from a height of 1 inch. The bulk density is then obtained by dividing the weight of the sample in gm by the final volume in cm^3 of the sample contained in the cylinder.

Tap density

A sample of about 50 cm3 of each powdered ingredients that was previously passed through a U. S. Standard no. 20 sieve is carefully introduced into a 100 ml graduated cylinder [12]. The cylinder is dropped at 2-sec intervals on a hard wooden surface hundred times from a height of 1 inch until no further decrease in the volume of powder takes place. The tap density is then obtained by dividing the weight of the sample in gm by the final volume in cm^3 of the sample contained in the cylinder.

Angle of repose

A glass funnel is held in place with a clamp on ring support over a glass plate. The glass plate is placed on a micro-lab jack. Approximately 100 g of powder is transferred in to the funnel (that was previously passed through a number 10 mesh size), keeping the orifice of funnel blocked by the thumb. As the thumb is removed, the lab –jack is adjusted so as to lower the plate and maintain about 6.4 mm gap between the bottom of funnel stem and top of the powder pile. When the powder is emptied from the funnel, the angle of the heap to the horizontal plane is measured with a protector [12]. Measure the height of the pile (h) and the radius of the base(r) with the ruler. The angle of repose is thus estimated by following formula.

$$\Phi = \tan^{-1}(h/r)$$

Hausner Ratio

The Hausner ratio is calculated by the formula given below, where ρB is the freely settled bulk density of the powder, and ρT is the tapped density of the powder [13].

$$H = \frac{\rho_T}{\rho_B}$$

Carr index

The Carr index is an indication of the compressibility of a powder. It is calculated by the following formula, where ϱB is the freely settled bulk density of the powder, and ϱT is the tapped density of the powder (Gibson, 2001).

$$C = 100 \times \left(1 - \frac{\rho_B}{\rho_T}\right)$$

Preparation of calibration curve for Piperine

Standard solutions of piperine were prepared within the concentration range 2-10 µg/ml in 10 ml volumetric flasks [13]. The absorbance of the piperine solution is measured at 342.5 nm (Figure 1) against ethanol and a calibration curve plotted (Figure 2).

RESULTS AND DISCUSSION

Physicochemical properties

Table-I shows the moisture content of Cyperus rotundus, Piper longum, Aconitum heterophy, Pistacia integerrima, lab formulation and marketed formulation were found respectively. The moisture content of formulation was within acceptable range (5-8%) thus implying that the formulation can be stored for a long period and would not easily be attacked by microbes. Physical properties namely tapped density, bulk density, angle of repose, hausner ratio and carr's index were calculated for Lab formulation, marketed formulation and its raw materials. The value of angle of repose for raw materials *Cyperus rotundus, Piper longum, Aconitum heterophy, Pistacia integerrima,* lab formulation and marketed formulation were 28.62, 31.28, 29.86, 24.34, 27.36, and 27.45 respectively which shows good flow properties of prepared lab formulation. The flow properties are also confirmed by Hausner's ratio and Carr's index (Table-1). Values of Hausner's ratio less than 1.25 indicate good flow (20% Carr Index) and the value greater then 1.25 indicates poor flow (33% Carr Index) (Gupta et al 2010). Both parameters were determined for prepared Ayurvedic formulation and it was found 1.25 and 20% respectively and indicates good flow characteristics.

Phytochemical analysis

Results of the phytochemical screening of the raw materials, lab formulation and marketed formulation of Balacaturbhadrika churna are concluded in Table-II. One notable difference as a result of the methods of extraction is the possibility that the alkaloids in *Piper longum* and *Pistacia integerrima* are more soluble in ethanol, the reason why the presence of that group was not detectable in the aqueous extract. Furthermore, where more than one test was conducted for the detection of a

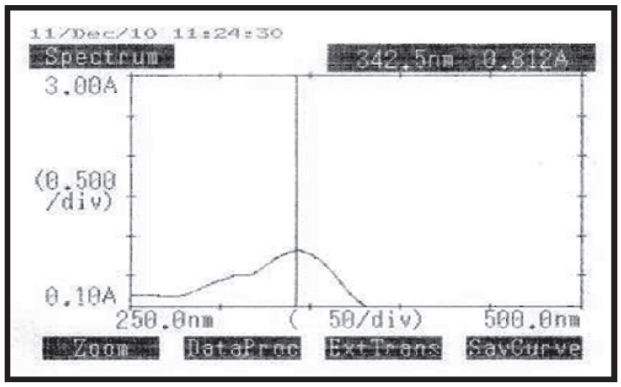

Figure 1: UV scan of piperine in ethanol

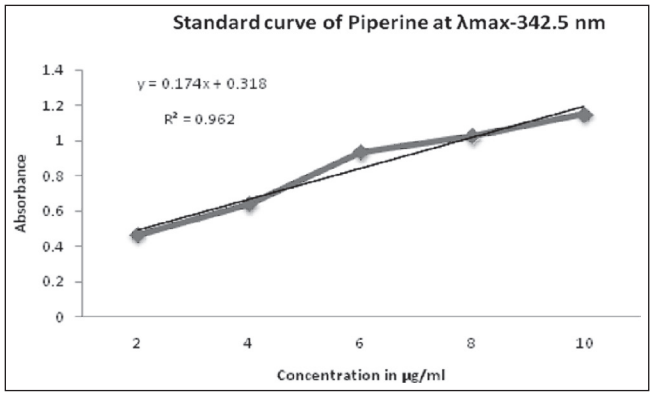

Figure 2: Standard curve of piperine in ethanol at 342.5 nm

chemical group such as the alkaloids, no differences in the results were observed for the different tests.

Out of the nine phytochemical groups investigated, seven namely carbohydrate, glycosides, tannins, flavonoids, fixed oil and proteins were detected in the ethanolic extract of lab and marketed formulations however the aqueous extracts of both formulations shows the presence of saponins with previously stated seven phytochemical groups. Steroids were absent in all the ingredients and formulations for both methods of extraction.

Determination of Ash value

Total ash value of *Cyperus rotundus, Piper longum, Aconitum heterophy, Pistacia integerrima,* lab formulation and marketed formulation were 7.346±0.346, 5.032±0.624, 2.981±0.243, 4.621±0.334, 8.148±0.337 and 19.633±0.552 respectively (Table-III). The value of total ash in marketed formulation is comparatively high in comparison to lab formulation may be because of the higher amounts of inorganic components present in marketed formulation. Acid-insoluble ash value of prepared lab formulations were 3.281 ± 0.286 and 5.041 ± 0.368 for lab and marketed formulation respectively shows that a small amount of the inorganic component is insoluble in acid it indicates adulteration of raw ingredients by substance like silica, rice husk is very less in both formulation. Low acid-insoluble ash value may also affect amount of the component absorbed in the gastrointestinal canal when taken orally.

Determination of extractive values

Alcohol-soluble and water soluble extractive values of ingredients and formulation are depicted in Table-IV, which shows 39.294 ± 2.226 and 30.662 ± 0.472 alcohol-soluble extractive value for lab and marketed formulation respectively which is higher than water soluble extractive value of both

Table 1: Physicochemical parameters of Balacaturbhadrika churna and its raw material

S.N.	Name	% LOD	Tap density	Bulk density	Angle of repose	Hausner ratio	Carr's index
1	CR	6.32±0.268	0.62	0.50	28.62	1.24	20
2	PL	7.78±0.642	0.66	0.52	31.28	1.26	21
3	AH	5.29±0.382	0.60	0.50	29.86	1.20	17
4	PI	8.02±0.196	0.52	0.41	24.34	1.26	21
5	LF	6.84±0.224	0.50	0.40	27.36	1.25	20
6	MF	5.88±0.292	0.62	0.52	27.45	1.19	16

CR (*Cyperus rotundus*), PL (*Piper longum*), AH (*Aconitum heterophy*), PI (*Pistacia integerrima*), LF (lab formulation), MF (Marketed formulation)

Table 2: Phytochemical characterization of ethanolic and aqueous extracts of Balacaturbhadrika churna and its raw materials

Test	Ethanolic extract						Aqueous extract					
	CR	PL	AH	PI	LF	MF	CR	PL	AH	PI	LF	MF
Alkaloids	+	+	+	+	+	+	+	–	+	–	+	+
Carbohydrates	+	+	+	+	+	+	+	+	+	+	+	+
Glycosides	–	+	–	+	+	+	+	+	+	+	+	+
Tannins and phenolic compounds	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	–	+	+	+	+	–	–	+	+	-	+
Fixed oil	+	–	+	–	+	+	–	–	–	+	+	+
Saponins	–	–	–	–	–	–	+	–	+	–	+	+
Proteins and amino acids	+	–	+	+	+	+	+	–	+	+	+	+
Steroids	–	–	–	–	–	–	–	–	–	–	–	–

CR (*Cyperus rotundus*), PL (*Piper longum*), AH (*Aconitum heterophy*), PI (*Pistacia integerrima*), LF (lab formulation), MF (Marketed formulation)

Table 3: Percentage ash value of Balacaturbhadrika churna and its raw materials

SN	Name	Total ash (% w/w)	Acid insoluble ash (% w/w)	Water soluble ash (% w/w)
1	CR	7.346 ± 0.346	0.756 ± 0.031	13.431 ± 0.387
2	PL	5.032 ± 0.624	1.302 ± 0.346	23.163 ± 0.736
3	AH	2.981 ± 0.243	1.324 ± 0.078	38.263 ± 0.642
4	PI	4.621 ± 0.334	2.418 ± 0.249	18.725 ± 0.354
5	LF	8.148 ± 0.337	3.281 ± 0.286	45.602 ± 0.414
6	MF	19.633 ± 0.552	5.041 ± 0.368	51.403 ± 0.223

CR (*Cyperus rotundus*), PL (*Piper longum*), AH (*Aconitum heterophy*), PI (*Pistacia integerrima*), LF (lab formulation), MF (Marketed formulation)

Table 4: Extractive values and Volatile contents of Balacaturbhadrika churna and its raw materials

Name	Alcohol-soluble extractive	Water-soluble extractive
CR	19.482 ± 0.468	24.268 ± 0.442
PL	28.282 ± 0.368	12.246 ± 2.638
AH	37.442 ± 2.664	20.842 ± 2.425
PI	48.726 ± 1.263	12.856 ± 1.424
LF	39.294 ± 2.226	20.224 ± 0.682
MF	30.662 ± 0.472	19.331 ± 1.552

CR (*Cyperus rotundus*), PL (*Piper longum*), AH (*Aconitum heterophy*), PI (*Pistacia integerrima*), LF (lab formulation), MF (Marketed formulation)

Table 5: Spectrophotometric determination of piperine content

Name	Piperine Content (% w/w ± SD)
Piper longum	0.695 ± 0.012 %
LF-1	0.392 ± 0.030 %
LF-2	0.363 ± 0.001 %
LF-3	0.382 ± 0.004 %
MF	0.233 ± 0.002 %

LF: lab formulation, MF: marketed formulation, P<0.001 (highly significant)

formulations. Higher ethanol-soluble extractive value implies that ethanol is a better solvent of extraction for the formulation than water.

Spectrophotometric analysis of piperine

The determination of formulations was carried out through UV spectrophotometer at 342.5 nm for piperine. The absorbance characteristics show that piperine follow Beer Lambert's law within the concentration range 2-10 µg/ml at the λ-max of 342.5 nm. The estimation of piperine content of the balchaturbhadrika churna and powder of Piper longum (Pippali) was carried out separately. The concentration of piperine content in raw material was found to be 0.695 ± 0.012 w/w in Piper longum. The content of piperine in different batches of balchaturbhadrika churna was found to be 0.392 ± 0.030 %, 0.363 ± 0.001 %, 0.382 ± 0.004 % and 0.233 ± 0.002 % w/w respectively for lab formulation (LF-1, LF-2, LF-3) and marketed formulation (MF) (Table-V). The developed method was found to be reliable, accurate, precise and sensitive.

CONCLUSION

WHO has emphasized the need to ensure quality control of Ayurvedic formulations by using modern technique and by applying suitable parameters and standards (WHO, 2007). It is the cardinal responsibility of the regulatory authorities to ensure that the consumers get the medication, with purity, safety, potency and efficacy. As prescribed by the WHO, evaluations of physicochemical and phytochemical properties are essential to standardize the different Ayurvedic formulations. In this connection authors investigated the stated parameters of an Ayurvedic formulation Balacaturbhadrika churna, which is amazingly unexplored till date. At the same time widely used by the ayurvedic medical practitioner for the treatment of various abdominal disorders. These explorations definitely help to set a standard of this traditional medicine.

ACKNOWLEDGEMENT

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Pharmacognostic Evaluation of *Cordia macleodii* Hook. Stem Bark

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ABSTRACT

Cordia macleodii Hook. (Boraginaceae), known as *Sikari/Phanki* by the tribals of Orissa, is a plant of ethnomedicinal importance and is available in Gandhamardana hills ranges of Orissa. Stem bark of this plant is used for healing wounds and for treating jaundice. In this study, an attempt has been made to study pharmacognostical characters of the stem bark which includes its macroscopic and microscopic characters and preliminary phytochemistry including TLC and HPTLC. Bark shows microscopic characters like cork, cortex, medullary rays, sclerenchyma fibres, phloem, cambium and crystals. The phytochemical tests show presence of alkaloids, glycosides, tannins and HPTLC profile shows presence of 9 spots and 8 spots at 254 and 366 nm respectively.

Key words: *Cordia macleodii*, ethnomedicine, pharmacognosy, jaundice, wound healing, Gandhamardan hill.

INTRODUCTION

Cordia macleodii Hook. (Boraginaceae), native to India, is a small sized tree. It has been reported that the tribal people use this plant as an aphrodisiac and also to treat mouth sores and jaundice[1]. Leaf of this plant is being reported as a wonderful wound healing drug[2]. Researches have been carried out to evaluate hepatoprotective activity[3], pharmacognostical evaluation[4] and pharmacological evaluation of wound healing activity[5] of its leaf. Though the stem bark of this plant has been highlighted for different enthnopharmacological properties[6] research study report on its stem bark, except its phytochemical constituents[7], is lacking[8]. In the present study an attempt has been made to evaluate the pharmacognostical characters of its stem bark.

Materials and Methods

Stem bark of *C. macleodii* Hook was used as material.

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Collection of Sample

The plant was identified by local traditional practitioners and authenticated by expert taxonomist, on the basis of characters given in Flora of Orissa[9]. Stem bark of the plant was collected from its natural habitat in the month of November and shade dried. The shade dried bark was pulverized and sieved through 80 mesh and preserved in an airtight glass container for future physicochemical and phytochemical anlysis.

Preservation of wet sample

Fresh stem bark sample was preserved in a solution prepared from glacial acetic acid, alcohol, formalin and distilled water following standard procedures[10].

Microscopic evaluation

Thin sections of the stem bark portion were taken by maceration method[11]. They were treated with respective reagents for detection of chemicals[12].

Photographs

Photomicrographs were taken by using Canon digital camera attached to Zeiss microscope.

Physicochemical investigation

The preserved sample as mentioned above was used for the physicochemical and preliminary phytochemical investigations by the standard procedure adopted by Ayurvedic Pharmacopoeia of India[13]. Fluorescence analysis was done as per Chase and Pratt (1949)[14].

HPTLC

Sample preparation

Hydrolyzed alcohol extract was used which was collected by Soxhlet extraction process. The filtrate was filtered and evaporated to dryness and the residue was dissolved in hydro alcohol.

Mobile phase : Ethyl acetate: Methanol: Water [100:17.5:12.5]
Stationary phase : Precoated Silica gel G_{254} (Merck)
Detection : (1) Day light
(2) Short U.V. (254 nm)
(3) Long U.V. (366 nm)
(4) Spraying with 10% Methanolic KOH.

RESULTS AND DISCUSSION

Morphology[15],[16] (Plate 1)

It is a small tree of 9-12 m. height, trunk about 50-60 cm in diameter. Bark is light green, 12-15mm thick; curved inside with longitudinal striations along with large amount of fibres, reddish brown colour inside, cut surface darker in colour. On injury, it forms exudate which is reddish brown in colour. Branchlets are white tomentose.

Microscopic Study

T. S. of stem bark: (Plate 2)

Bark brownish with a thickness of 12-15 mm shows a well developed outer cork which comprises about 1/3rd of the thickness of entire bark and the remaining portion is composed of a very thin/undifferentiated cortex and a very wide phloem which occupies the major portion of the bark followed by a narrow cambium.

Cork (Fig.2a)

Cork is made of 8-12 layers of rectangular shaped tangentially elongated radially arranged cells with brownish colouring materials in 5-10 rows.

Cortex(Fig.2a)

Cortex is very narrow and cannot be differentiated from the inner phloem.

Medullary rays (Fig.2e)

Wide medullary rays start just below from the narrow cortex and reach up to the region of cambium. The medullary ray cells are mostly radially elongated, rectangular shaped cells and bi to multiseriate. Most of the medullary ray cells are multiseriate and 3-5 cells wide and several of them also contain prismatic crystals of calcium oxalate. One or two large groups of sclerenchyma cells are also found in this region. They also contain prismatic crystals of calcium oxalate and brownish contents.

Crystals (Fig.2c)

Some of the parenchyma cells surrounding them contain prismatic crystals of calcium oxalate embedded with brownish colouring materials.

Parenchyma(Fig.2e)

Middle bark is narrow and comprised of a thin phalloderm consisting of thin walled parenchyma.

Inner bark (Fig.2d)

Inner bark occupies the major part of the living tissue which constitutes the phloem, medullary rays and tangential rows of sclerenchyma fibres in groups. Outer zone consists of many radial as well as tangential strips of compressed parenchyma cells associated with phloem tissues and group of sclerenchyma fibres and secretory cells containing brown contents and prismatic crystals of calcium oxalate. Many radial rows of crushed phloem elements are also present on either sides of radially running medullary rays.

Phloem (Fig.2g)

Phloem is formed by a large number of fibre groups arranged in tangential rows alternating with parenchyma and phloem tissues. The fibre cells are much thick walled and lignified. Most of the fibre groups are rectangular in outline and each group contains 30-80 fibre cells and rarely 100-150 cells may also be seen. The cells of the phloem alternating with these fibre groups are smaller in dimension and thin walled with regularly arranged radial rows. Secretory cells filled with brown contents and prismatic crystals are common throughout this region.

Sclerenchyma fibres (Fig.2e)

Fibre groups are lignified and on application with phloroglucinol followed by HCl show reddish/pinkish colour formation establishing all of them are lignified sclerenchyma fibres. In the phloem region, some of the parenchyma cells are also found with pitted walls and some of the cells are also found filled with orange reddish contents.

Cambium

A cambium is present just inner to the phloem region. The cambium is made up of thin walled rectangular shaped cells arranged in radial rows.

Starch grains

Few simple and compound starch grains are also found in parenchyma cells particularly phloem region. On application of iodine shows blue colouration indicating the presence of starch in few of parenchyma cells.

Physicochemical tests

Physicochemical analysis of the leaf shows the following results and could be taken as standard for further study. Loss on drying shows the moisture of the drug which was found to be 9.21% w/w. Total ash content shows the presence of inorganic materials in the drug. Acid insoluble ash was

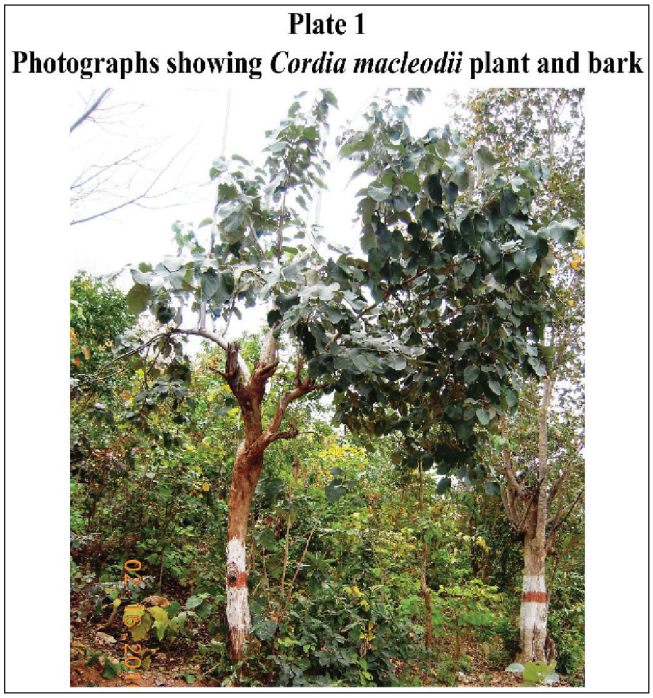

Figure 1a: *C. macleodii* plant

Figure 1a: *C. macleodii* bark

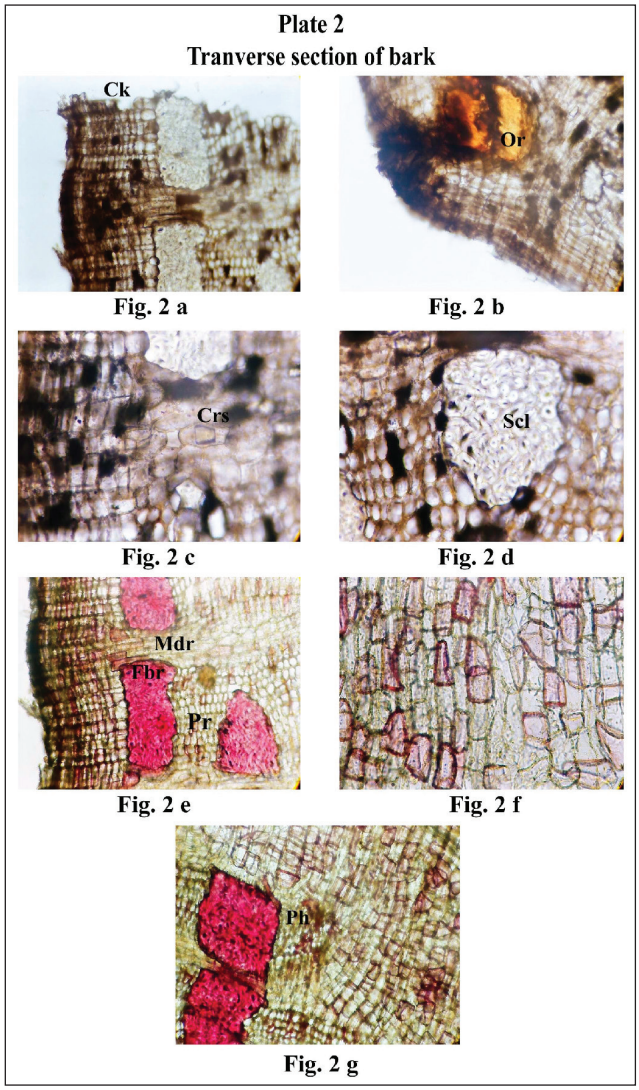

Figure 2a: Cork
Figure 2b: Cortex
Figure 2c: Crystals
Figure 2d: Sclerenchyma fibres
Figure 2e: Parenchyma
Figure 2f: Inner bark
Figure 2g: Phloem

found to be 0.73% w/w. Water soluble and alcohol soluble extractive values denote the percentage of that can mix with the solvents which were found as given in the table1. Qualitative tests show presence of alkaloids, glycosides, phenols, flavonoids, terpenoids and tannin. HPTLC of the leaf powder shows 9 spots under short UV (254 nm) while it shows 8 spots under long UV (366nm) radiation.

CONCLUSION

Table No. 3 shows 9 spots when scanned at 254 nm and 8 spot at 366 nm. After spraying with 10% FeCl3 it shows 8 spots. This spray reagent is used for Phenolic and Flavonoid type compound which means Phenolic and Flavonoid type compounds are present in drug sample.

On the basis of the pharmacognostical characters bark of *Cordia macleodii* Hook. can be identified and its identity, purity and strength can be assessed.

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Table 1: Physicochemical analysis *C.macleodii* bark

Parameters	Stem bark
Foreign Matter	Nil
Loss on Drying % w/w	9.21
Total Ash Content % w/w	11.07
Acid Insoluble Ash % w/w	0.73
Water Soluble Extractive Value % w/w	7.8
Alcohol Soluble Extractive Value % w/w	13.75
pH	6.01

Table 2: Qualitative tests of *C.macleodii* bark

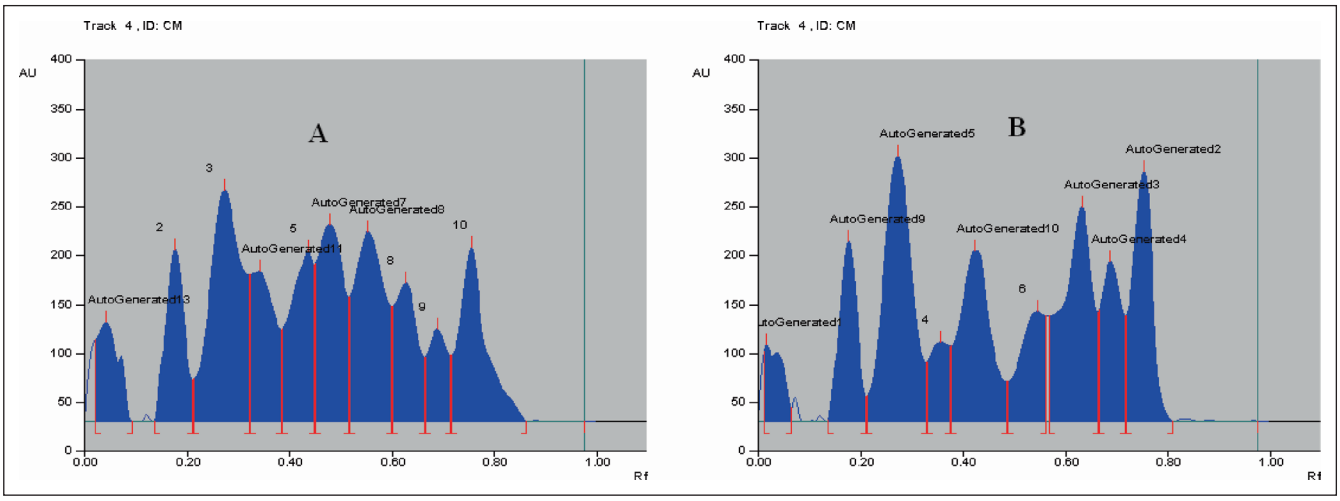
Parameters	Stem Bark
Alkaloids (M.E.)	+
Glycosides (W.E.)	+
Phenols (M.E.)	+
Flavanoid (M.E.)	+
Terpenoids (Cl.E.)	+
Tannin (W.E.)	+
Saponin (W.E.)	–

M.E. – Methanol extract W.E. – Water extract

Table 3: Showing consolidated data of HPTLC profile of *C.macleodii* bark

Conditions	No. of spots	Max. R_f
Short UV (254 nm)	9	0.18,0.27,0.34,0.44,0.48,0.55,0.63,0.67,0.76
Long UV (366 nm)	8	0.18,0.27,0.36,0.42,0.55,0.63,0.69,0.75
After spray with 10% Fecl3	8	0.18,0.27,0.44,0.48,0.55,0.63,0.67,0.76

Diagram 1: Showing HPTLC profile of *C. macleodii* bark (A-254 nm, B-366nm)



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Phytochemical Screening and *in vitro* Antioxidant Activity of Jawarish Amla- A Poly Herbal Formulation

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ABSTRACT

The present research work was carried out to study the antioxidant potential of methanolic extract of Jawarish amla using various *in vitro* tests including Reducing power assay, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, DPPH free radical scavenging assay, and hydrogen peroxide method. These various antioxidant activities were compared to standard ascorbic acid. Preliminary phytochemical screening revealed that the extract of jawarish amla possesses flavonoids, steroids and phenolic compounds. In this study, quantitative determinations of flavonoids and phenols were conducted by colorimetric methods, using aluminum chloride method and Folin Ciocalteu reagent respectively. The results indicated that methanolic extract of Jawarish amla possess antioxidant property.

Key words: Antioxidant activity, Ascorbic acid, DPPH, Jawarish Amla

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) are often generated as byproducts of biological reactions or from exogenous factors.[1] These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells [2] including DNA, if excess ROS are not eliminated by antioxidant system. They play important roles in aging and in the pathogenesis of age related disorders such as cancer, hypertension, atherogenesis, Alzheimers disease, and Parkinsons disease.[3-5] Recent investigations have shown that the antioxidants with free-radical scavenging properties of plant origins could have great importance as therapeutic agents in aging process and free radical mediated diseases including neuro degeneration.[6,7] Plant extracts[8] and plant products such as flavonoids and other polyphenolic constituents have been reported to be effective radical scavengers and inhibitors of lipid peroxidation.[9, 10] Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant. In view of its wide use and its chemical composition, the methanolic extract of Jawarish amla was evaluated for its *in vitro* anti oxidative activities.

Biological studies of plant extracts have been carried out to verify the pharmacological properties of the plants. The radical scavenging, reducing capacity and metal chelating properties of antioxidants are known to eliminate and prevent the generation of free radical. The properties have been contributing directly or indirectly in the prevention of pathogenesis and deterioration of food,[11, 12] whereas the ability of plant extract to kill or inhibit the growth of microorganisms is at interest for the development of antimicrobial agent. Thus, such studies add value and provide scientific information to continually validate the potential of the plant known as ethnomedicine.[12, 13]

Jawarish amla name is due to its chief ingredient amla (*Emblica officinalis*) belongs to family Euphorbiaceae. It is extensively found all over India, as well as Sri Lanka, Malaysia, China, Pakistan and Bangladesh.

The fresh (or) the dry fruit is used in traditional medicines for the treatment of diarrhoea, jaundice and inflammations.[14] The pulp of the fruit is smeared on the head to dispel headache and dizziness.[15] Amla leaves and fruit have been used for fever and inflammatory treatments by rural

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populations in its growing areas. The earlier study have demonstrated potent anti-microbial,[16] adaptogenic,[17] anti-tumour[18] and anti-ulcerogenic activities.[19] Amla is one of the richest sources of Vitamin C. In addition to this, potent antioxidant, several active tannoid principles (Emblicannin A, Emblicannin B, Punigluconin and Pedunculagin) have been identified which appear to account for its health benefits.[20, 21] Earlier work on this plant showed the occurrence of tannins, lignans, flavonoids and alkaloids.[22-24]

MATERIALS AND METHODS

Chemical and reagents

Folin Ciocalteu reagent, 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH), Nitro blue tetrazolium (NBT), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. Catechin and rutin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade available commercially.

Preparation of extract

The formulation of Jawarish amla was extracted with mixture of methanol by a soxhlet apparatus at 60ºC. The solvent was completely removed by rotary evaporator (Rotavapor® R-210, BUCHI Corporation) and obtained brownish gummy exudates. This crude extract was used for further investigation for antioxidant properties.

Phytochemical screening

The freshly prepared extract of Jawarish amla was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using standard procedures.[25, 26]

Determination of total phenolic content

Total phenols were determined by Folin Ciocalteu reagent.[27] A dilute extract of formulation (0.5 ml of 10mg/ml) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm (Schimadzu UV-Vis 1601). The standard curve was prepared using 25, 50, 100, 150, 200, 250, 300 µg/ml solutions of gallic acid in methanol.

Determination of total flavonoid content

Aluminum chloride colorimetric method was used for flavonoids determination.[28] Formulation extract (0.5 ml of 10mg/ml) in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm (Schimadzu UV-Vis 1601). The calibration curve was prepared by preparing rutin solutions at concentrations 10 to 100 µg/ml in methanol.

In vitro Antioxidant Parameter

Reducing power assay

The reducing power of Jawarish amla was determined according to the method described.[29] Different concentrations of Jawarish amla extract (10 µg/ml – 50 µg/ml) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml. 0.1%) and the absorbance was measured at 700 nm (Schimadzu UV-Vis 1601). Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6) was used as blank solution.

Superoxide anion scavenging activity assay

The scavenging activity of the Jawarish amla towards superoxide anion radicals was measured by the method.[30] Superoxide anions were generated in a non-enzymatidc phenazine methosulfate nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of phosphate buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations (10 µg/ml – 50 µg/ml) of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm (Schimadzu UV-Vis 1601) was measured in spectrophotometer. The super oxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction.[31] Reaction mixture contained 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2, and 1.5 ml of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm (Schimadzu UV-Vis 1601) was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method.[32] Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of Jawarish amla extract at various concentrations (10 µg/ml – 50 µg/ml) and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm. The nitric oxide radicals scavenging activity was calculated.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

The free radical scavenging capacity of the extracts was determined using DPPH.[33] DPPH solution (0.004% w/v) was prepared in 95% methanol. Methanolic extract of Jawarish amla, was mixed with 95% methanol to prepare the stock solution (1 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes then Jawarish amla extract was added followed by serial dilutions (10 µg/ml to 50 µg/ml) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Schimadzu UV-Vis 1601). Ascorbic acid was used as a reference standard and dissolved in double distilled water to make the stock solution with the same concentration (1 mg/ml) followed by serial dilutions (10 µg/ml to 50 µg/ml). Control sample was prepared containing the same volume without any extract and reference ascorbic acid.

$$\% \text{ Inhibition} = [(A_0\text{-}A_1) / A_0 \times 100]$$

Where, A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance of the extract or standard.

Scavenging of Hydrogen Peroxide

The ability of Jawarish amla to scavenge hydrogen peroxide was determined according to the method.[34] A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV-Vis 1601). Jawarish amla extract (5 µg/ml – 25 µg/ml) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 2 mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 20 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of Jawarish amla and standard compounds was calculated by using the above equation.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical screening of the extract of Jawarish amla revealed the presence of various bioactive components of which flavonoids and tannins were the most prominent and the result of phytochemical test has been summarized in the Table I.

Total phenolic content

Phenolic compounds are known to be powerful chain breaking antioxidants and are important constituents of plants. Phenolic compounds may contribute directly to antioxidative action. It is suggested that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1.0 gm daily from a diet rich in fruits and vegetables. The total phenolic content of the jawarish amla extract measured by Folin-Ciocalteu

Table 1: Phytochemical screening of methanolic extract of Jawarish Amla

Phytochemical screening of methanolic extract of Jawarish Amla	
Alkaloid	+
Sterol	+
Carbohydrate	+
Phenolic compound	+
Flavonoid	+
Proteins and amino acids	+
Lipid/fat	-
Mucilage	+
Resin	+

Table 2: Total phenolic and flavonoid content of Jawarish Amla extract

Total phenolic content (µg/mL)	Mean± SEM	Total flavonoid content (µg/mL)	Mean ± SEM
102.83		132.25	
98.16	103.33 ± 3.139	135.87	135.83 ± 2.055
109.00		139.37	

reagent in terms of gallic acid equivalent jawarish amla extract was 103.33±3.139µg/mL (Table 2).

Total flavonoid content

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process.[35, 36] Therefore, in the present study, total flavonoid content present in extract was estimated using Aluminum chloride colorimetric method. In Jawarish amla extract, the flavonoid content was found to be 135.83±2.055µg/ml (Table 2).

In vitro Antioxidant Activity

Reducing power assay

Figure 1 shows the reducing power of the jawarish amla extract and ascorbic acid, as a function of their concentrations. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers causes the reduction of the Fe 3+/ferricyanide complex to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration. The reducing properties are generally associated with the presence of reductones,[37] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[34]

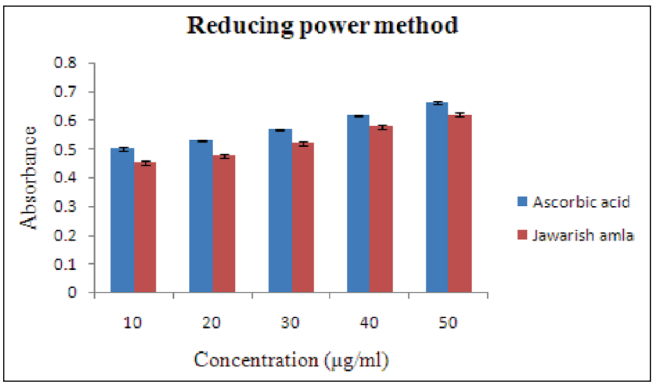

Figure 1: Reducing power assay

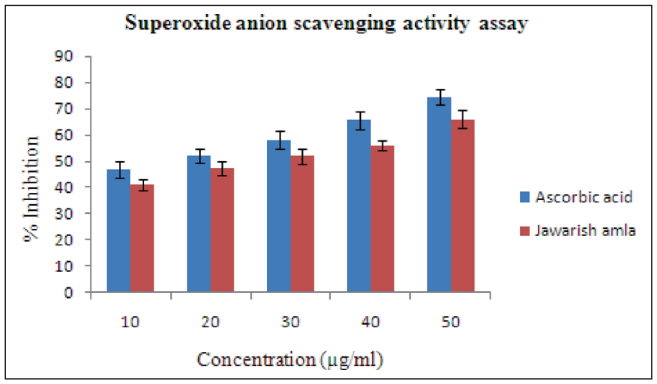

Figure 2: Superoxide anion scavenging activity assay

Superoxide anion scavenging activity assay

It is well known that superoxide anions damage biomolecules directly or indirectly by forming H_2O_2, OH, peroxy nitrite or singlet oxygen during aging and pathological events such as ischemic reperfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation.[38]The superoxide anion radical scavenging activity of Jawarish amla extract assayed by the PMS-NADH system was shown in figure 2. The superoxide scavenging activity of Jawarish amla extract was increased markedly with the increase in concentrations. Thus, higher inhibitory effects of the rhizomes extracts on superoxide anion formation noted herein possibly renders them as a promising antioxidants. The half inhibition concentration (IC_{50}) of Jawarish amla extract was 45.94µg/ml while IC_{50} value for ascorbic acid was 25.83µg/ml. These results suggested that Jawarish amla extract has a potent superoxide radical scavenging effects.

Hydroxyl radical scavenging activity assay

Activity of the rhizomes extract on hydroxyl radical has been shown in figure 3. Hydroxyl radical is highly reactive oxygen centered radical formed from the reaction of various

hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecule. Jawarish amla extract exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. The IC_{50} value of extract was found to be 6.59µg/ml while IC_{50} value for ascorbic acid was 1.27µg/ml.

Nitric oxide scavenging activity assay

The jawarish amla extract showed a moderate nitric oxide-scavenging activity between 10 and 50 µg/ml in a dosedependent manner (IC_{50} = 22.00 µg/ml) (Figure 4). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions.[39] The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. The extract showed a moderate nitric oxide-scavenging activity. The % inhibition was increased with increasing concentration of the extract. The IC_{50} value of ascorbic acid was found to be 3.90µg/ml.

Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

The jawarish amla extract showed a concentration-dependent antioxidant activity by inhibiting DPPH radical with an IC_{50} value of 11.07µg/ml (Figure 5). The IC_{50} value of ascorbic acid was found to be 7.79µg/ml, which was used as standard. The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenyl picrylhydrazine. It has been found that ascorbic acid, reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability.[40] It appears that the jawarish amla extract possesses hydrogen donating capabilities and acts as an antioxidant. The scavenging effect increased with increasing concentration of the extract.

Scavenging of Hydrogen Peroxide

As shown in figure 6, Jawarish amla extract also demonstrated hydrogen peroxide decomposition activity in a concentration dependent manner with an IC_{50} of 6.05µg/ml while IC_{50} value for ascorbic acid was 2.60µg/ml. Hydrogen peroxide

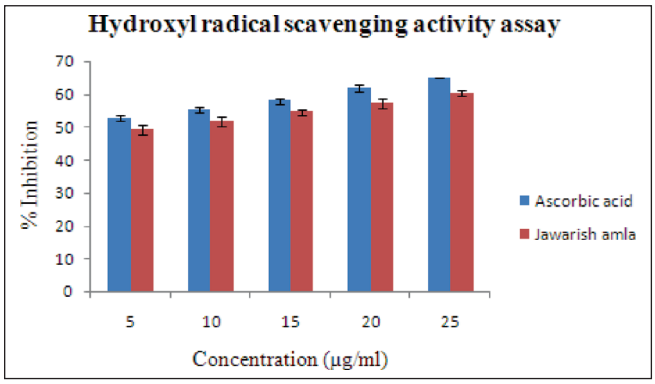

Figure 3: Hydroxyl radical scavenging activity assay

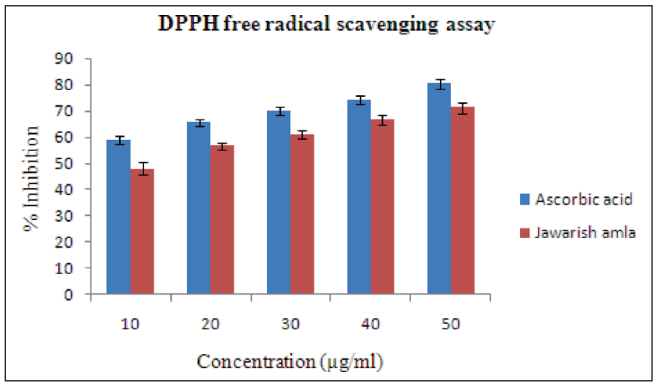

Figure 5: Scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical

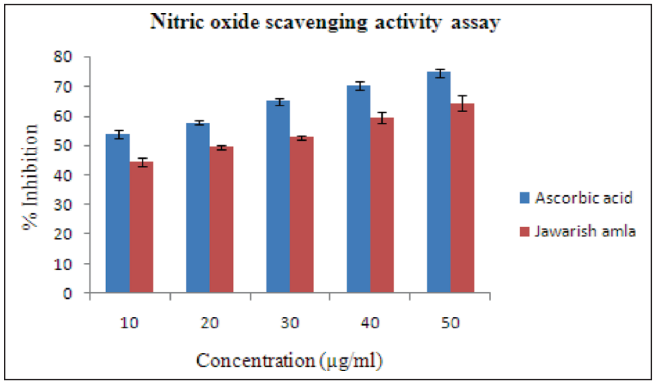

Figure 4: Nitric oxide scavenging activity assay

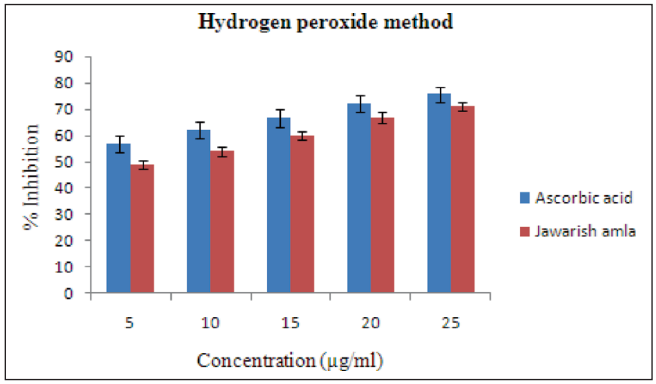

Figure 6: Scavenging of Hydrogen Peroxide

is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form hydroxyl radical and this may be the origin of many of its toxic effects.[41] It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The decomposition of H_2O_2 by Jawarish amla extract may at least partly result from its antioxidant and free radical scavenging activity.

CONCLUSION

For the purpose of characterizing antioxidant activity of plant extracts, it is desirable to subject it to a battery of tests that evaluates the range of activities such as Reducing power assay, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, DPPH free radical scavenging assay, and hydrogen peroxide method in formulation of jawarish amla. The *in vitro* antioxidant activities of the methanolic extract indicated the efficacy of the formulation as a source of natural antioxidants which will have application towards reducing lipid peroxidation /oxidative stress with consequent health benefits. The activity of jawarish amla might be due to the presence of amla which is a well known plant antioxidant.[39] Further research work is required for isolation and characterization of phytoconstituents responsible for its antioxidant property.

ACKNOWLEDGEMENT

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Phytochemical Investigation of Methanolic Extract of *Cassia fistula* Leaves

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ABSTRACT

Introduction: Recently there has been a shift in universal trend from synthetic to herbal medicine, which we can say' Return to Nature'. *Cassia fistula* or golden shower plant has plenty of medicinal values. This paper represent the isolation of two new chemical entities, present the leaves of cassia fistula and their medicinal aspects. **Methods:** Leaves of *Cassia fistula* was used as a plant material. Extraction was done with methanol followed by isolation procedure was done using HPTLC. Confirmation of compounds was done using UV, FTIR, NMR and MASS spectroscopy. *In vitro* antibacterial screening was carried out by disc diffusion method. **Results:** Air dried powdered leaves of *Cassia fistula* were extracted using methanol. From this extract two compounds were isolated in their pure form using Column chromatography and HPTLC. Identification of these two isolated compounds was confirmed by physico-chemical data, spectral interpretation and elemental analysis. **Conclusions:** This is the first report of the isolation of the 2 new compounds from *cassia fistula* leaves extracted in methanol extract. These compounds are seen to exhibit moderate antimicrobial property.

Key words: Antimicrobial activity, HPTLC, MASS, NMR,

INTRODUCTION

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and is becoming an important cause of morbidity and mortality in immune compromised patients in developing countries.[1] In recent years, attempts have been made to investigate the indigenous drugs against infectious diseases. This may help to develop safer antimicrobial drugs.[2]

Cassia fistula (family: *Fabaceae*), is commonly known as Amaltaash phal,[3-4] has been reported to possess laxative, Antioxidant, Hepatoprotective and Antiviral activity etc.[5-6] *Cassia fistula* has traditionally been used to treat leprosy, tuberculosis, syphilis, rheumatism, skin disease.[7] The Ayurvedic pharmacopoeia of India indicated the fruit pulp for constipation, colic, chlorosis and urinary disorders.[8] The fruit of *Cassia fistula* is used to treat diabetes.[9] *Cassia fistula* plant is rarely ever wholly leafless, but in some localities it is almost bare between March and May and new leaves appear during April-July. The flowers appear along with the leaves, in dry areas the flowers however appear till October.[10]

The various chemical constituents isolated from the plant are fistucacidin, leucocyanidin, leucopelargonidin, hexacosanol, lupeol, Physcion, Rhein glycoside, Kaempferol, Chrysophanol, Sennoside A, Sennoside B, Quercetin, Epicatechin, Procyanidin B2, Stigmasterol and β-sitosterol.[11-13] Owing to their varied bioactivities exhibited by *Cassia fistula*, efforts have been made from time to time to generate libraries of these isolated compounds and screen them for potential biological activities.

MATERIALS AND METHODS

Plant Material

The plant material (Leaves) of *Cassia fistula* was collected from Mathura, Uttar Pradesh, India in the month of January 2008. It was identified by NISCAIR, New Delhi, Ref. no. NISCAIR/RHMD/Consult/2008-09/1145/177. Leaves of *Cassia fistula* were dried in shade and reduced to coarse powder for extraction, isolation and characterization of chemical constituents.

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Extraction

Leaves of cassia fistula were coarsely powdered in a suitable grinder. These powdered leaves were poured in glass container and sufficient quantity of acetone solution was added in this container. Stir the solution until a nice dark green liquid was achieved then separate the green colored chlorophyll-acetone solution from coarse powdered leaves using separating funnel.[14-15] 5 kg chlorophyll free powdered leaves of *Cassia fistula* were extracted with 10 liter of methanol in a Soxhlet extractor for 48 hr. After complete extraction, the extract was concentrated using a rotary evaporator to afford a yellowish mass.[15] Prepared yellowish mass was then subjected to various spectral analytical techniques for the identification of the individual compounds.

Isolation of the individual compounds

Silica gel (60-120 mesh) was used as absorbent for column chromatography. The column was taken and packed with glass wool at the bottom of the column. The slurry was prepared using silica gel and hexane. It was poured slowly from the top of the column in a little quantity allowing for the even and uniform packing. 2/3rd of the column was packed by using above procedure. The extract was dissolved in the minimum quantity of hexane and chromatographed over silica gel.[16] It was then eluted with different solvents in increasing order of polarity viz. hexane, petroleum ether (40-60 °C), benzene, chloroform, ethyl acetate, acetone and methanol. The fractions were collected and marked. The marked fractions were subjected to high performance thin layer chromatography to check homogeneity of various fractions using Toluene-ethyl acetate-formic acid-methanol (20:12:4:4) as mobile phase and Aluminium foil silica gel 60 F_{254} column.

HPTLC analysis for marked fractions

A densitometric HPTLC analysis was performed for the development of characteristic finger printing profile. The fraction which was obtained from methanolic extract of *cassia fistula* leaves was dissolved with HPLC grade methanol 100 mg/0.5ml. The solution was centrifuged at 3000 rpm for 5 min and used for HPTLC analysis. Then, 2 µl of the samples were loaded as 7 mm band length in the 10 x 10 Silica gel 60F TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase (Toluene-ethyl acetate-formic acid-methanol 20:12:4:4) up to 90 mm. The developed plate was dried using hot air to evaporate solvents from the plate and sprayed with stannic chloride reagent.[17] The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at UV 280 nm. [Figure 1, 2]

Identification of the isolated individual compounds

IR spectrums of compounds were recorded on a Perkin Elmer Spectrum RXI FTIR system by using potassium bromide pellets.

^{1}HNMR spectra of the compounds were recorded on Bruker NMR spectrophotometer in DMSO using TMS as internal standard.

For Mass spectra compounds were dissolved in dissolving solvents and then diluted with water containing 0.1% formic acid and 80% acetonitrile before injection into the MS system mass charge was adjusted at some values.

Antimicrobial activity

Media

Nutrient agar media (Difco laboratories) pH 7.2, nutrient broth media (Difco laboratories) pH 6.8, Sabouraud dextrose agar media (Biolife Vole Monza) pH 5.6 were used for antibacterial screening, antifungal screening and MIC determination respectively.[18]

Antibacterial screening

In vitro antibacterial screening was carried out by disc diffusion method.[19-22] which is a qualitative to semi-quantitative test. Briefly, 20 ml quantities of nutrient agar were plated in Petri dish, solidified and inoculated with 0.1 ml of standardized inoculum of each bacterial culture by swabbing over the agar surface. Filter paper discs (6 mm in diameter) impregnated with various concentrations of Compound 1 and Compound 2 was placed on test organism inoculated plates. Dimethylsuphoxide (DMSO) was used to dissolve the compound and was completely evaporated before application on test organism inoculated plates. Blank disc impregnated with solvent DMSO followed by drying off was used as negative control. The activity was determined after 24 hr. of incubation at 37°C. The diameter of zone of inhibition produced by the Compound 1 and Compound 2 was then compared with the standard antibiotic ciprofloxacin 2 µg/disc. Sample was used in triplicate for the determination of antibacterial activity.

Antifungal screening

This was also carried out by disc diffusion method [23-26]. In this method 20 ml of Sabouraud dextrose was plated in Petri dish, solidified and inoculated with 0.1 ml of standardized inoculum of each fungal culture by swabbing

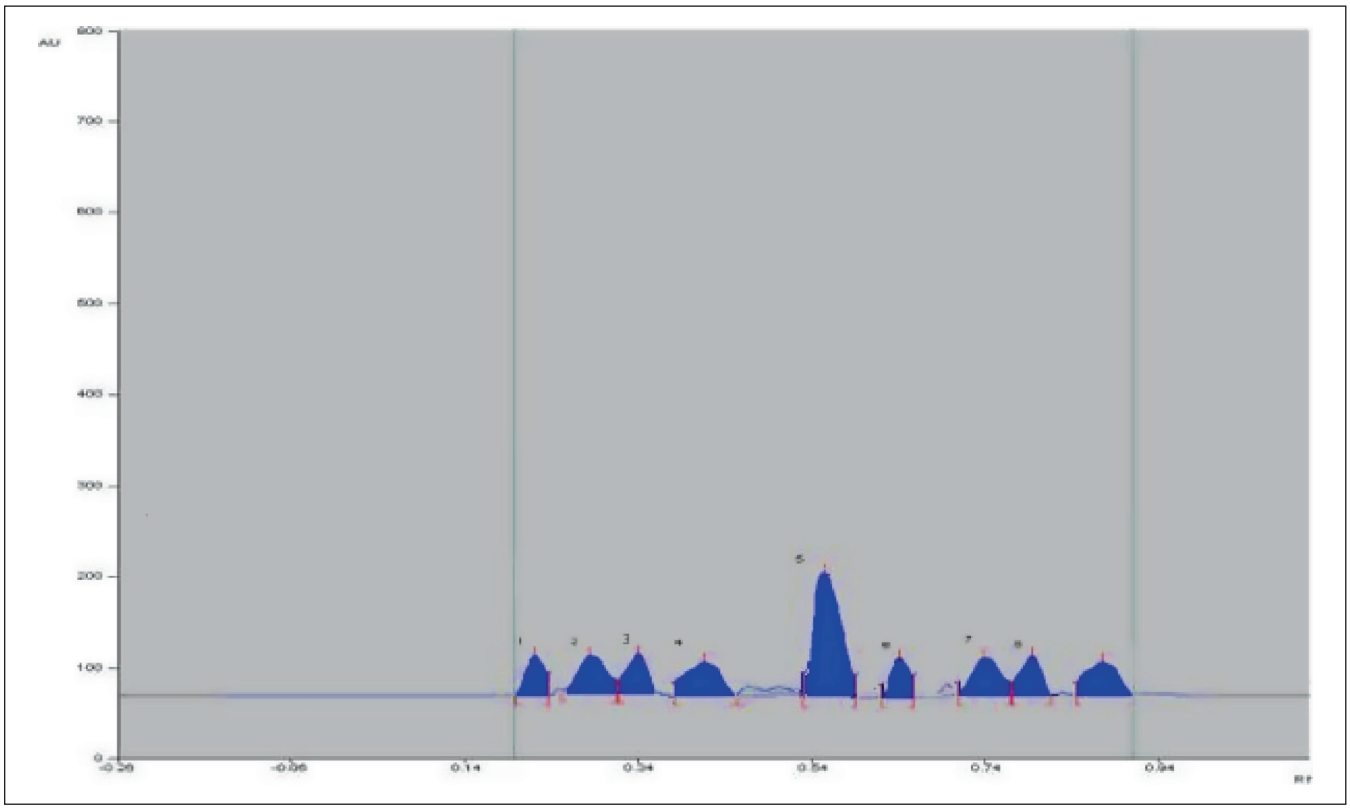

Figure 1: HPTLC finger print profile of methanol extract of Cassia fistula Linn. rf 0.55

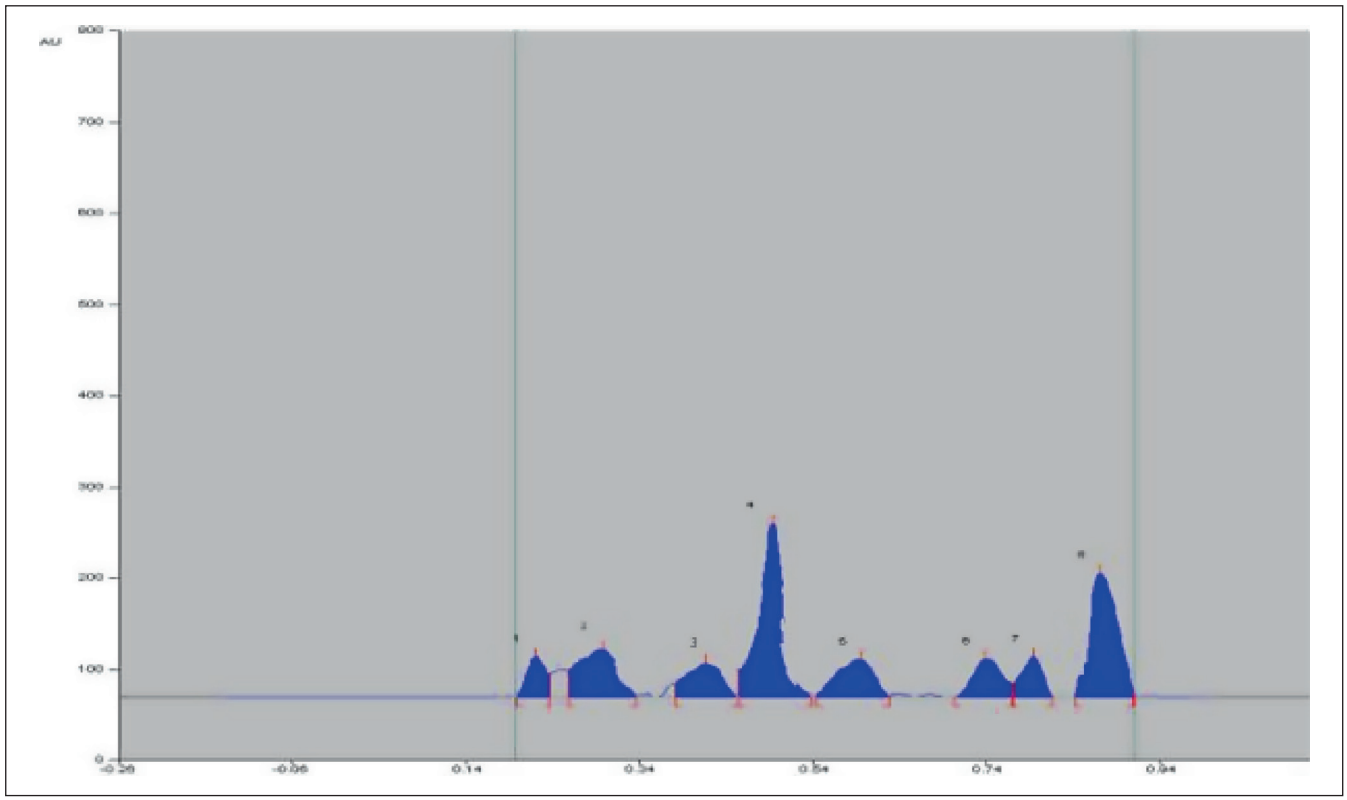

Figure 2: HPTLC finger print profile of methanol extract of Cassia fistula Linn. rf 0.45

over the agar surface. The diameter of zone of inhibition produced by the Compound 1 and Compound 2 was then compared with the standard antifungal drug fluconazole 2 µg/disc. Sample was used in triplicate for the determination of antifungal activity.

Minimum inhibitory concentration (MIC) determination

Stock solution of 400 µl/ml of the Compound 1 and Compound 2 was prepared followed by 2 ml of the stock solution was added into 2 ml of previously sterilized double strength nutrient broth and mixed well. From this 2 ml was transferred to another test tube containing 2 ml of normal strength nutrient broth. The serial dilution was done in this manner till 6.25 µl/ml concentration of Compound 1 and Compound 2 was reached. Then 50 µl of standardized inoculum of the bacteria was added into each tube and kept for incubation at optimum temperature i.e. 37±5°C for 24 hr. and examined for growth visually. The MIC of Compound 1 and Compound 2 was taken as the lowest concentration that showed no growth. Growth was observed in those tubes where the concentration of the Compound 1 and Compound 2 was below the inhibitory level and the broth medium was turbid. The nutrient broth media with three drops of Tween 80 and ciprofloxacin were used as negative and positive control, respectively for antibacterial activity and Tween 80 and Fluconazole used as a negative and positive controle, respectively for antifungal activity.[27-31]

RESULTS

Two new compounds were isolated first time from methanolic extract of cassia fistula leaves had following properties-

Compounds 1:

R_f value: 0.55, **Empirical formula:** $C_{13}H_{14}O_{5,}$ **m.pt.:** 208-210°C

UV: λmax(DMSO): 277 nm (log ε 4.18); **FTIR (KBr):** 3590.49 (O-H str.), 3560.62 (O-H str. Ar), 3067.82 (C-H str. Ar), 2982.60 (C-H str.), 1731.52 (C=O str.), 1582.94 (C=C str.), 1238.21 (C-O str.), 900.70 cm^{-1} (C-C str.); **^{1}HNMR (DMSO-d_6) :** δ 1.165-1.167 (d, 3H, CH_3), 1.252-1.292 (d, 2H, CH_2), 1.411 (s, 2H, CH_2), 1.502-1.588 (m, 1H, CH), 2.868 (s, 1H, OH, exchangeable with D_2O), 3.068 (s, 1H, OH, exchangeable with D_2O), 5.588 (s, 1H, OH exchangeable with D_2O), 7.270(s,1H, Ar-H),7.281(s,1H, Ar-H),7.295 ppm (s,1H, Ar-H); **ESI full mass-MS: m/z (%)** 251(19)$[M+1]^+$, 252(11)$[M+2]^+$, 250(100) $[M]^+$, 234(40) $[M\text{-}CH_3]^+$, 219(19)$[M\text{-}OCH_3]^+$, 191(29)$[M\text{-}C_3H_7O]^+$, 161(28), 151(12), 124(16), 101(10), 59(20);

Elemental Analysis: Calculated: C 62.93, H 5.64; Found: C 62.90, H 5.60%

It was confirmed that compound 1 was (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl)chromone had the following structure [Figure 3].

Compounds 2:

R_f value: 0.45, **Empirical formula:** $C_{23}H_{30}O_{10}$**, m.pt.:** 250-252°C

Empirical formula- $C_{23}H_{30}O_{10,}$ m.pt.-

UV: λmax(DMSO): 269 nm (log ε 4.30); **FTIR (KBr):** 3611.27 (O-H str.), 3074.90 (C-H str. Ar), 2919.60 (C-H str.), 1711.52 (C=O str.), 1632.52 (C=C str.), 1257.82 (C-O str.), 970.70 cm^{-1} (C-C str.); **^{1}HNMR (DMSO-d_6):** δ 2.122 (s, 2H, CH_2), 2.432-2.455 (d, 2H, CH_2), 2.766-2.821 (m, 5H, CH), 3.706 (s, 6H, OCH_3), 3.884 (s, 4H, OH, exchangeable with D_2O), 7.281-7.414 ppm (m, 7H, Ar-H); **ESI full mass-MS: m/z (%)** 451(28)$[M+1]^+$, 452(11) $[M+2]^+$, 450(100) $[M]^+$, 419(22)$[M\text{-}CH_3O]^+$, 315(39), 277(21) $[M\text{-}C_6H_6O_6]^+$, 163(15), 135(12), 91(49), 77 (28), 66(20), 51(19);

Elemental Analysis: Calculated: C 58.66, H 5.82; Found: C 58.63, H 5.80 %

It was confirmed that compound 2 was Benzyl-2β-O-D-glucopyranosyl-3,6-dimethoxybenzoate had the following structure [Figure 4]

The results of antibacterial activity of Compound 1 and Compound 2 against the test bacteria are presented in tables [Table 1, 2]. In comparison to reference standard ciprofloxacin (2 µg/disc), Compound 1 and Compound 2 exhibited significant antibacterial activity at 10 µg/disc [Figure 5].

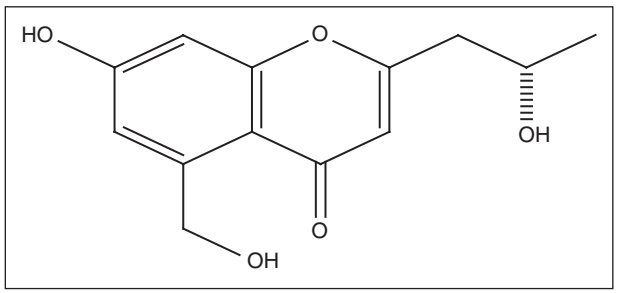

Figure 3: Structure of compound 1

Compound 1 showed highest activity against *Staphylococcus aureus* and lowest activity against *Klebseilla pneumonia* [Figure 6]. Compound 2 showed highest activity against *Bacillus subtilis* and lowest activity against *Pseudomonas aeruginosa* [Figure 7].

The MIC values of compound 1 against Gram-positive and Gram-negative ranged from 22 to 38 and 41 to 50 µg/ml, respectively [Table 3]. The MIC values for compound 2 against gram-positive and gram-negative ranged from 29 to 37 and 31 to 50 µg/ml, respectively [Table 4].

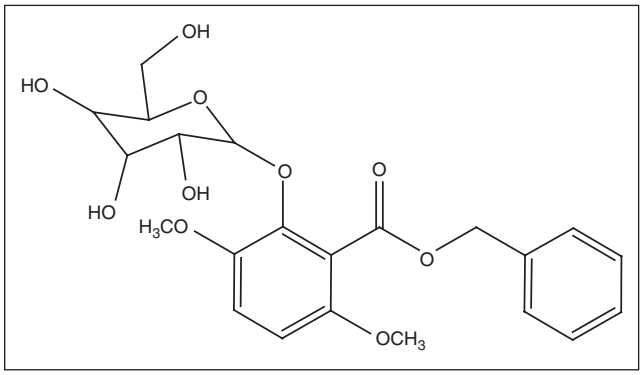

Figure 4: Structure of compound 2

In comparison to reference standard fluconazole (2 µg/disc), Compound 1 and Compound 2 exhibited significant antifungal activity against *Candida albicans*, *Aspergillus niger, Penicillium chrysogenum and Sacromyces cerevisiae* at 10 µg/disc [Table 5, 6].

Compound 1 showed highest activity against *Sacromyces cerevisiae* and lowest activity against *Penicillium chrysogenum* [Figure 8]. Compound 2 showed highest activity against *Sacromyces cerevisiae* and lowest activity against *Aspergillus niger* [Figure 9]. The MIC values of compound 1 and compound 2 against used fungal strains ranged from 18 to 27 µg/ml [Table 7] and 22 to 28 µg/ml [Table 8].

DISCUSSION AND CONCLUSION

Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modelling.[32, 33] Isolation of (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl)chromone (compound 1) and Benzyl-2β-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) from *Cassia fistula* Leaves extracted in methanol and its antibacterial and antifungal

Table 1: *In-vitro* antibacterial activity of (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl)chromone (compound 1) and Ciprofloxacin

Test organism	Zone of inhibition (diameter in mm)		
	Compound 1 (5µg/disc)	Compound 1 (10µg/disc)	Ciprofloxacin (2µg/disc)
	Gram-positive bacteria		
Staphylococcus aureus	6.86 ± 0.02	11.73 ± 0.04	16.56 ± 0.01
Bacillus subtilis	6.70 ± 0.04	11.70 ± 0.03	16.32 ± 0.01
	Gram-negative bacteria		
Escherichia coli	6.65 ± 0.03	11.53 ± 0.04	17.10 ± 0.02
Pseudomonas aeruginosa	5.56 ± 0.02	11.93 ± 0.04	17.13 ± 0.01
Klebseilla pneumonia	5.11 ± 0.01	10.32 ± 0.01	16.51 ± 0.02

The control disc used for solvent had no zone of inhibition; hence; this data has not been shown
Data shown in mean + SEM (n=3)

Table 2: *In-vitro* antibacterial activity of Benzyl-2′-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) and Ciprofloxacin

Test organism	Zone of inhibition (diameter in mm)		
	Compound 2 (5µg/disc)	Compound 2 (10µg/disc)	Ciprofloxacin (2µg/disc)
	Gram-positive bacteria		
Staphylococcus aureus	6.70 ± 0.02	12.85 ± 0.04	17.31 ± 0.01
Bacillus subtilis	6.95 ± 0.04	12.85 ± 0.04	17.62 ± 0.01
	Gram-negative bacteria		
Escherichia coli	6.55 ± 0.03	12.10 ± 0.04	17.10 ± 0.02
Pseudomonas aeruginosa	5.45 ± 0.02	10.30 ± 0.04	16.25 ± 0.01
Klebseilla pneumonia	5.90 ± 0.01	10.20 ±0.01	16.25 ± 0.02

The control disc used for solvent had no zone of inhibition
Data shown in mean + SEM (n=3)

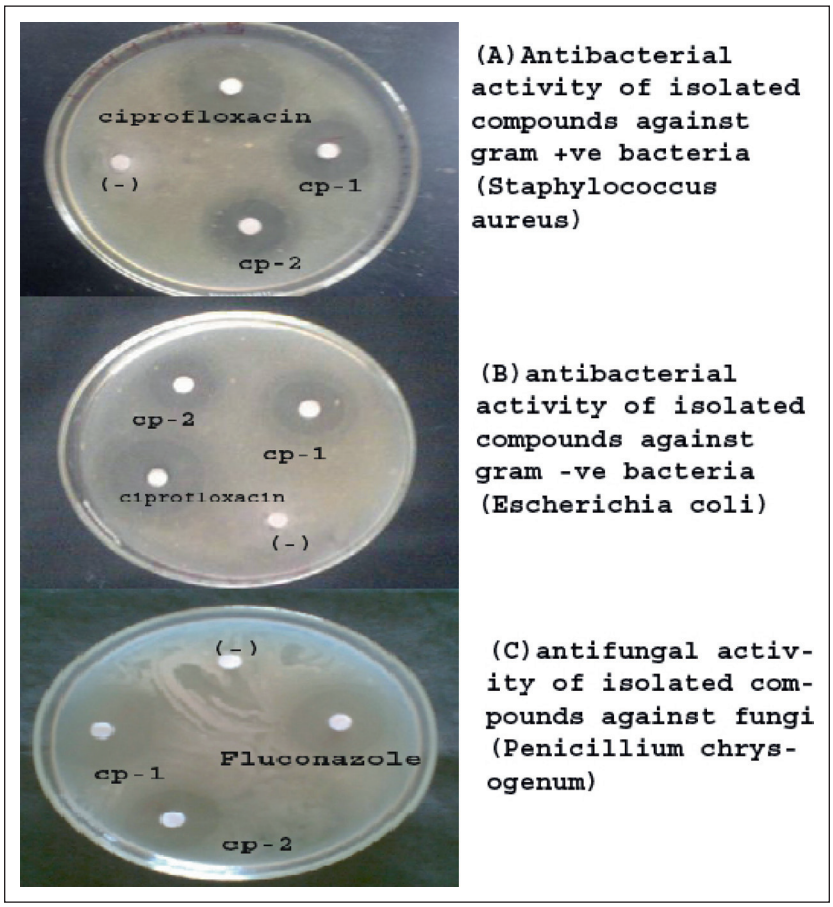

Figure 5: Antimicrobial activity of isolated compounds

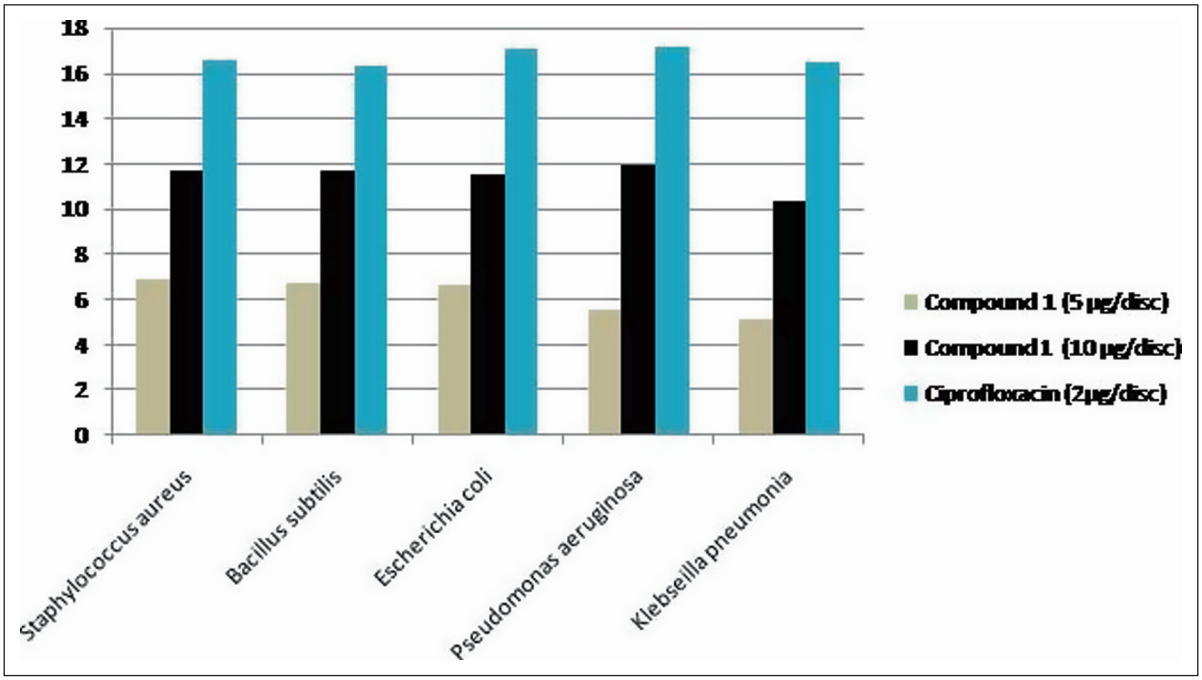

Figure 6: Antibacterial activity of compound 1 (5µg/disc and 10µg/disc) and Ciprofloxacin

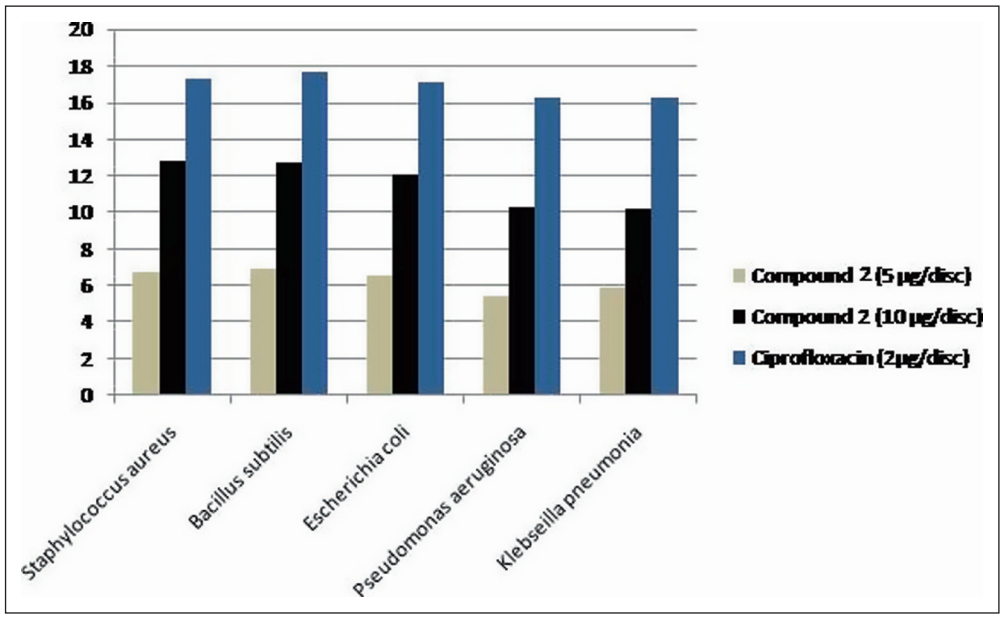

Figure 7: Antibacterial activity of compound 2 (5µg/disc and 10µg/disc) and Ciprofloxacin

Table 3: Minimum inhibitory concentration of (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl) chromone (compound 1) and Ciprofloxacin

Bacteria	MIC values of Compound 1 (µg/ml)	MIC values of Ciprofloxacin (µg/ml)
Staphylococcus aureus	22	5.0
Bacillus subtilis	38	0.35
Escherichia coli	41	2.0
Pseudomonas aeruginosa	32	2.5
Klebseilla pneumonia	50	7.0

The negative control containing solvent had no MIC value

Table 4: Minimum inhibitory concentration of Benzyl-2′-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) and Ciprofloxacin

Bacteria	MIC values of Compound 2 (µg/ml)	MIC values of Ciprofloxacin (µg/ml)
Staphylococcus aureus	29	5.0
Bacillus subtilis	37	0.55
Escherichia coli	43	2.5
Pseudomonas aeruginosa	31	2.0
Klebseilla pneumonia	50	7.3

The negative control containing solvent had no MIC value

Table 5: In *vitro* antifungal activity of (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl)chromone (compound 2) and Fluconazole

	Zone of inhibition (diameter in mm)		
Test organism	Compound 1 (5µg/disc)	Compound 1 (10µg/disc)	Fluconazole (2µg/disc)
Candida albicans	5.19 ± 0.02	11.50 ± 0.02	16.12 ± 0.02
Aspergillus niger	5.40 ± 0.02	11.32 ± 0.01	17.20 ± 0.02
Penicillium chrysogenum	6.21 ± 0.02	10.79 ± 0.04	17.11 ± 0.03
Sacromyces cerevisiae	5.30 ± 0.02	11.63 ± 0.02	16.37 ± 0.02

The control disc used for solvent had no zone of inhibition
Data shown in mean + SEM (n=3)

Table 6: In *vitro* antifungal activity of Benzyl-2′-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) and Fluconazole

	Zone of inhibition (diameter in mm)		
Test organism	Compound 2 (5µg/disc)	Compound 2 (10µg/disc)	Fluconazole (2µg/disc)
Candida albicans	5.39 ± 0.02	10.50 ± 0.02	16.32 ± 0.02
Aspergillus niger	5.80 ± 0.02	10.32 ± 0.01	17.80 ± 0.02
Penicillium chrysogenum	6.61 ± 0.02	10.70 ± 0.04	17.51 ± 0.03
Sacromyces cerevisiae	5.90 ± 0.02	12.63 ± 0.02	16.40±0.02

The control disc used for solvent had no zone of inhibition
Data shown in mean + SEM (n=3)

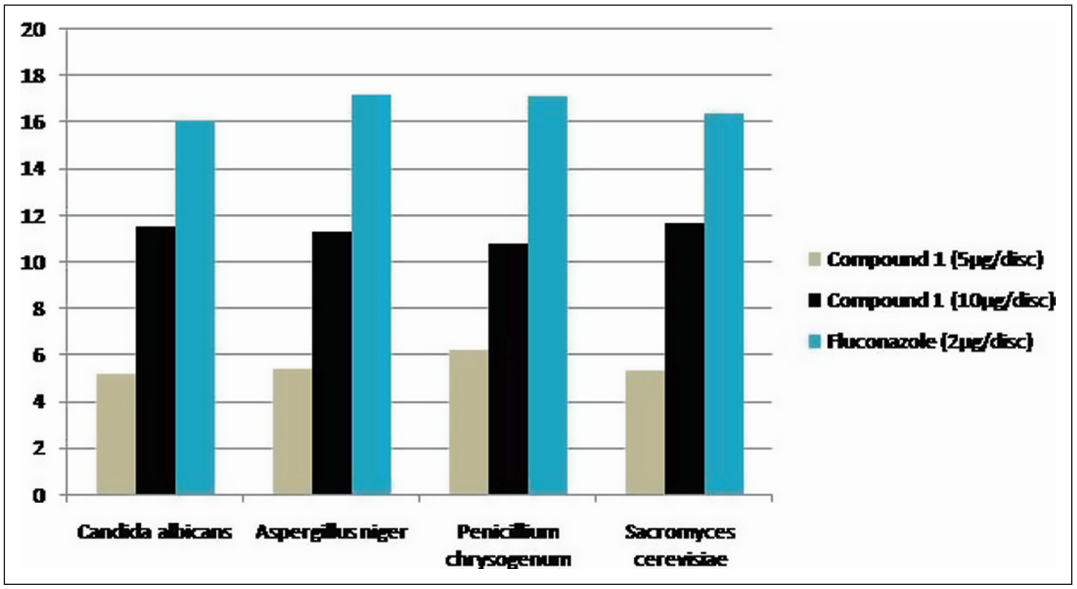

Figure 8: Antifungal activity of compound 1 (5µg/disc and 10µg/disc) and Fluconazole

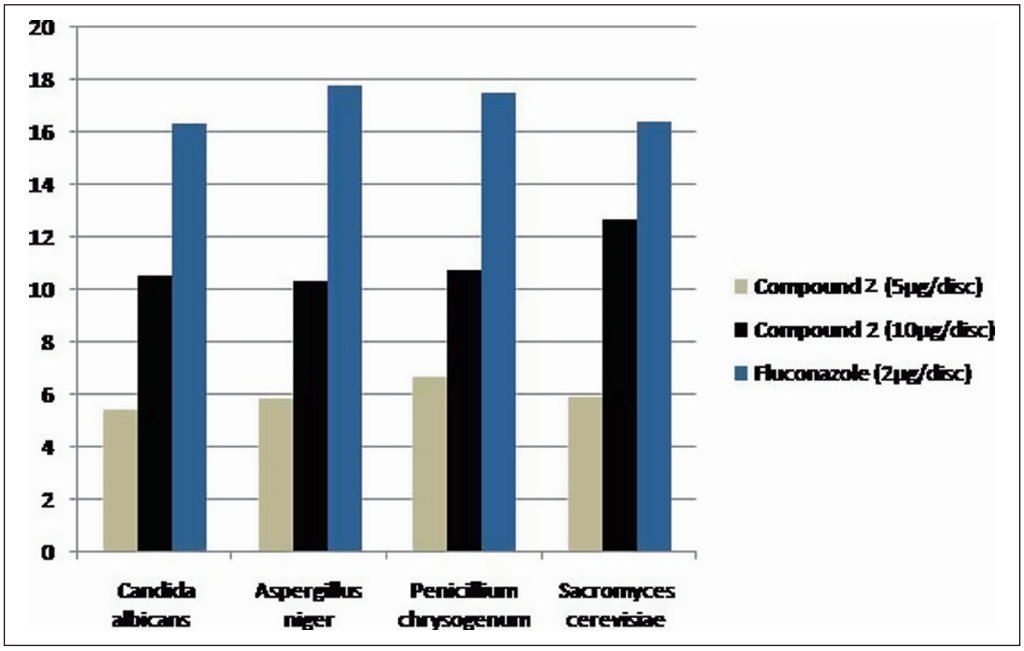

Figure 9: Antifungal activity of compound 2 (5µg/disc and 10µg/disc) and Fluconazole

Table 7: Minimum inhibitory concentration of (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl) chromone (compound 2) and Fluconazole

Fungi	MIC values of Compound 1 (µg/ml)	MIC values of Fluconazole (µg/ml)
Candida albicans	18	2.9
Aspergillus niger	26	2.0
Penicillium chrysogenum	27	1.6
Sacromyces cerevisiae	21	2.9

The negative control containing solvent had no MIC value

Table 8: Minimum inhibitory concentration of Benzyl-2′-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) and Fluconazole

Fungi	MIC values of Compound 2 (µg/ml)	MIC values of Fluconazole (µg/ml)
Candida albicans	22	2.9
Aspergillus niger	28	2.0
Penicillium chrysogenum	25	1.6
Sacromyces cerevisiae	25	2.9

The negative control containing solvent had no MIC value

studies are being for the first time reported now. HPTLC technique has been used for the very first time for the isolated compounds of leaves of *Cassia fistula.*

These compounds are seen to exhibit moderate antimicrobial property. Although (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl)chromone (compound 1) showed activity against all tested bacteria, it was better against Gram-positive bacteria than Gram-negative bacteria. In this study highest activity against *Staphylococcus aureus* and lowest activity against *Klebseilla pneumonia* was observed. Benzyl-2β-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) showed activity against all tested bacteria, it was better against Gram-positive bacteria than Gram-negative bacteria. In this study highest activity against *Bacillus subtilis* and lowest activity against *Pseudomonas aeruginosa* was observed. (2′S)-7-hydroxy-5-hydroxymethyl-2-(2′-hydroxypropyl) chromone (compound 1) and Benzyl-2β-O-D-glucopyranosyl-3,6-dimethoxybenzoate (compound 2) showed good antifungal activity against all tested fungi *Candida albicans*, *Aspergillus niger, Penicillium chrysogenum and Sacromyces cerevisiae.*

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Pharmacognostic and Phytochemical Evaluation of *Citrus aurantifolia* (Christm) Swingle PEEL

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ABSTRACT

Citrus aurantifolia (Christm) Swingle belongs to Family Rutaceae, is commonly known as Kagzi Nimbu or Acid lime. The peel is a leathery exocarp, or skin of the fruit containing numerous oil glands. Lime peel tea was traditionally used as an appetite stimulant, for cold and cough, for sore throat and as a digestive stimulant. It can also be use as an astringent for skin. Lime peel essential oil is use as an aroma and flavour enhancer. The present study deals with the pharmacognostic and preliminary phytochemical evaluation of *Citrus aurantifolia* (Christm) Swingle peel extract including macroscopic, microscopic characters, physiochemical evaluation and preliminary phytochemical study. Thin layer chromatography has also been performed. Microbial load of dried powder material was determined to avoid the contamination and for safety of crude drug. The present work will provide referential information for the correct identification and standardisation of the crude drug and will ensure the use of only genuine and uniform material in preparation of herbal formulation.

Key words: *Citrus aurantifolia* (Christm) Swingle, pharmacognosy studies, physicochemical analysis, TLC finger printing.

INTRODUCTION

Herbal medicines are currently in demand and their popularity is increasing day by day. In the healthcare sector, WHO recommends and encourages the use of traditional herbs or remedies because huge amount of raw material is easily available. Plants are very complex in nature. Their therapeutic activity varies according to species, geographical location and harvesting processes. Improper authentication of herbs, adulterations by microorganism, pesticide residue; has made standardization of herbal drug of primary importance. According to the World Health Organization, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing the identity and the degree of purity of such materials and should be carried out before any tests are undertaken.[1]

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Lime fruit (*Citrus aurantifolia* (Christm.) Swingle)

Scientific classification (Liogier, 1988)

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Sapindales
Family	:	Rutaceae
Genus	:	Citrus
Species	:	C. aurantifolia
Binomial name	:	*Citrus aurantifolia* (Christm.) Swingle
Common name	:	Acid Lime, Key lime (Mexican), Kagzi nimbu (Hindi)

It is an evergreen, spiny shrub or small tree 5 to 6 m in height. The plant has single or multiple stems and irregular branches covered with smoothish brown to gray bark. The twigs are quadrangular (when young), green, and bare sharp axillary spines 3 to 17 mm long. The leaves are yellow-green to dark green, with 5- to 28-mm winged petioles and elliptic to oval leathery 4- to 13-cm long blades with edges that have minute rounded teeth. The crushed foliage has a strong,

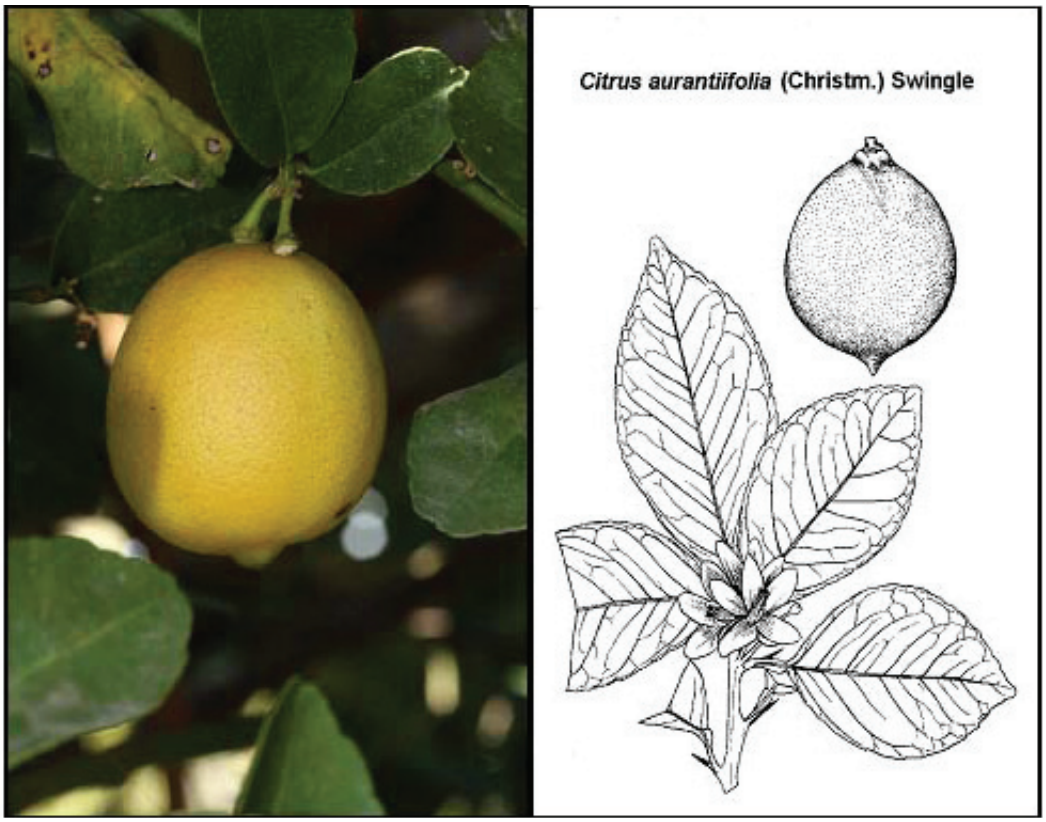


Figure 1: Citrus aurantifolia (Christm.) Swingle; T.S. of dried Citrus aurantifolia (Christm) Swingle Peel.

distinct, spicy (citrus) odor and taste. The four- to five-petaled white flowers occur in few-flowered axillary clusters. The fruits are ellipsoidal, 3 to 5 cm in diameter, have juicy, greenish-yellow flesh, and are yellow at maturity (*Fig. 1*). They contain a few white, pointed seeds about 1 cm long.[2-3]

MATERIALS AND METHODS

Collection and identification of plant material

Citrus aurantifolia (Christm) Swingle fruits were purchased from local market of Mumbai, India, and the botanical identity of plant was confirmed by Dr. C. S. Latto, University of Mumbai. Lime fruits were washed thoroughly under water. Peels were separated from fruits and dried under shade. Then the dried material subjected to the powder form and stored in an airtight container at room temperature.

Reagents and Chemicals

All reagents and chemicals used for testing were analytical grade obtained from Fisher Chemicals Ltd., Mumbai, SD Fine Chemicals Limited, Mumbai and Qualigens Chemicals, Mumbai.

Macroscopic and Microscopic analysis

The macroscopic characters of lime fruit peels were studied according to standard methods for its morphological characters such as color, odor, taste, shape, size, etc .and reported. Microscopic evaluation was carried out for transverse section of Lime peel and dreid powder material**.** The outer epidermal layer (in fragments) were cleared in chloral hydrate, stained with phloroglucinol and concentrated HCl and mounted with glycerin and observed under a compound microscope.

Physicochemical analysis

Physicochemical properties such as Total ash, Acid insoluble ash, Water soluble ash, were determined according to the specifications as per Indian Pharmacopeia. [4] Percentage of ash value (indicative of thepurity of the drug) and extractive values (represent the presence of polar and non polar compounds) were performed according the official methods. Water and alcohol soluble extractive values were estimated by cold maceration according to the method as prescribed by WHO.

Determination of volatile oil

The determination of volatile oil in peels was carried out by steam distillation according to the method of Khandelwal.

20 gm of chopped peels were taken into round bottomed flask and mixed with 300 ml of water: glycerin (1:1) then the Clevenger's apparatus was assembled. The round bottom flask was heated for 2 hrs and the oil was collected in the graduated tube. The oil was obtained from which percentage of oil in the peels of *Citrus aurantifolia* (Christm) Swingle was calculated.

Preliminary phytochemical analysis

The powdered plants were subjected to preliminary Phytochemical screening for qualitative detection of phytoconstituents. The dried and coarsely powder (50g) were extracted with n-Hexane (65°C-80°C) and Methanol (64.5°C-65.5°C) by Soxhlet extraction and by cold maceration. The extracts obtained were evaporated to dryness by Rota evaporator and weighed to calculate percentages in terms of initial air dried plant material. The extracts as mentioned above, were subjected to various qualitative phytochemical tests for the identification of chemical constituents present in the plant material according to the method of Harborne, 1998, Khandelwal 2002. [5-6]

Fluorescence Analysis of powder sample

When physical and chemical parameters are inadequate as it often happens with the powdered drugs, the plant material may be identified from their adulterants on basis of fluorescence study. It can be use as a diagnostic tool for testing the adulterations. Fluorescence characteristic of powdered drug with different reagent were observed under day light and U.V. light after drug treatment with different reagents as described by Ansari et al. 2006 [7]

Thin Layer Chromatography study

Accurately weighed 100 mg methanolic extract and n-hexane were diluted with 10 ml of methanol and n-hexane respectively and used for spotting the chromatographic plates. Silica Gel 60 F Plates were used as a stationary phase. Mobile phase was Toluene: Ethyl Acetate: Methanol (8.5:1.0:0.5). After development of TLC plates by one dimensional ascending method, visualization was performed by spraying with anisaldehyde sulphuric acid reagent followed heating at 100-120° C for 10 minutes and R_f values were calculated.

Micrbial load

To minimize the chance of contaminated finished product, it is necessary to control the microbial content of raw materials hence evaluation of the microbial load is very important Presence or absence of pathogen decides whether the raw material is suitable for preparation of finished product. First sample preparation was done in Soya bean -casein digestive broth. Eenumeration and presence or absence of pathogens was studied Standard plate count or viable count method is used for enumeration of bacteria, yeast and mold counts were determined as per the WHO guidelines.[8]

RESULTS AND DISCUSSION

Macroscopic and Microscopic analysis

Macroscopic characters of fresh and dried lime peel vary. Color of outer surface of fresh peel is Bright Green to Yellow and turns to Brownish Yellow when dried. Inner surface of fresh peel is white and turn to Whitish Brown after drying. Fresh Peel has strong and aromatic odor and dried peels are not very strong and less aromatic. Taste is sour and bitter. Shape of fruits is globose or ovoid. Dried peels are triangular to spiral shaped. These brittle strips are about 1 to 2 cm long, 2 to 2.5 cm broad and 0.1 to 0.2 cm in thickness, externally rugose, marked with numerous minute pits which represent oil glands.

T.S. of peel shows a layer of epidermis, below this lies the 2-3 rows of hypodermis which is composed of small parenchymatous cells. Underneath the hypodermis there is a layer of mesocarp cells which are large and porous. Big oval shape oil glands are embedded in the cells of mesocarp and below the hypodermis. *(Fig.2A, 2B)*

The powder characters of a drug are mainly used in the identification of the drug in the powder form. Powder also showed the presence of spiral vessels and fragments of broken oil glands. *(Fig.3A, 3B)*

Determination of physicochemical parameters

The physical-chemical parameters are mainly used in judging the purity and quality of the drug. Ash values of a drug give an idea of the earthy matter or inorganic composition. Water soluble ash value is more than acid insoluble ash. Extractive values give an idea about the chemical constituents present in the drug as well as useful in the determination of exhausted or adulterated drugs. The results suggest that the powdered drug have high water soluble extractive value. *(Table1)*

Determination of volatile oil

Volatile oils are characterized by their odor, oil-like appearance and ability to volatilize at room temperature. Aromatic compounds predominate in certain volatile oils.

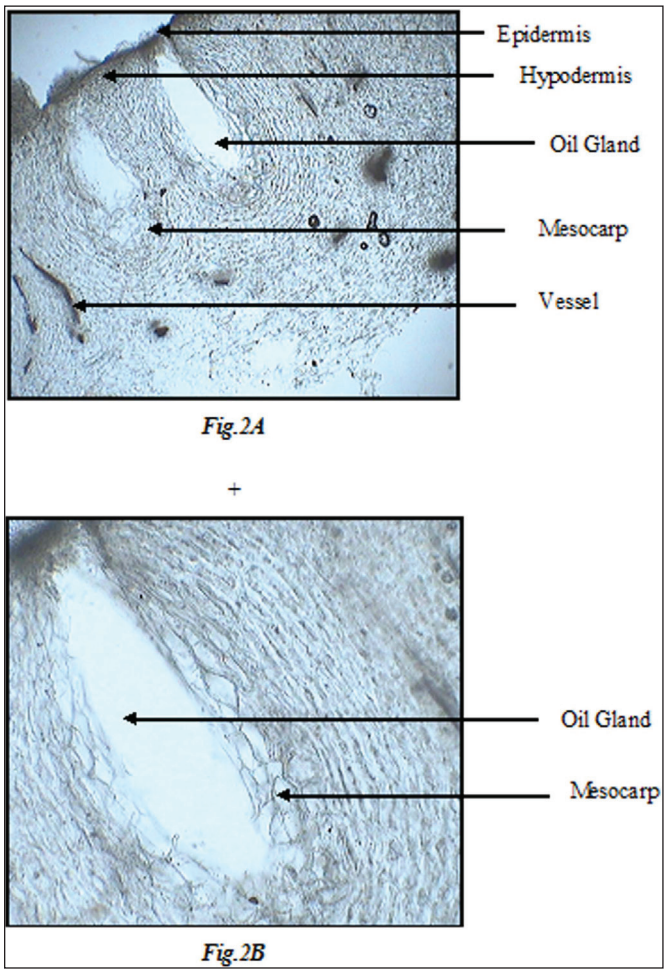


Figure 2A: Epidermis, Hypodermis, Oil Gland, Vessels in T.S. of dried Citrus aurantifolia (Christm) Swingle Peel.
Figure 2B: Oil Gland, Mesocrp in T.S. of dried Citrus aurantifolia (Christm) Swingle Peel.

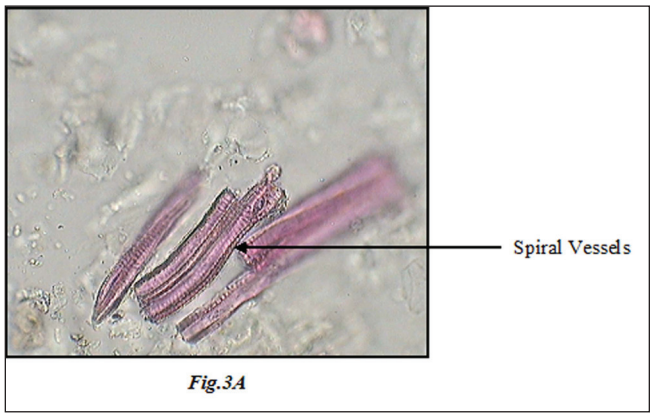
Table1: Physicochemical analysis of dried peel powder of *Citrus aurantifolia* (Christm) Swingle.

Parameter	Average Value
Total ash content	4.03%
Acid insoluble ash	0.06%
Water soluble ash	0.72%
Alcohol soluble extractive	12.35%
Water soluble extractive	31.28%

Lime peel volatile oil has charcteristic smell. The percentage of oil in the peels of *Citrus aurantifolia* (Christm) Swingle was calculated as 1.42%

Determination of percentage yield of extracts in different solvents

Percentage yield of all extracts such as hot methanolic extract (HME), Cold methanolic extract (CME), Hot n-hexane extract (HHE) and Cold n-hexane extract (CHE) of *Citrus aurantifolia* (Christm) Swingle is calculated and indicated in *(Table2)*



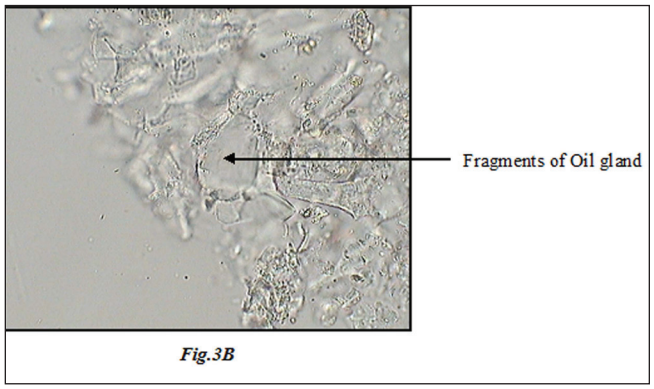


Figure 3A: Spiral Vessels in dried powder of Citrus aurantifolia (Christm) Swingle Peel
Figure 3B: Fragments of Oil glands in dried powder of Citrus aurantifolia (Christm) Swingle Peel

Table 2: Percentage yield determination of *Citrus aurantifolia* (Christm) Swingle peel extracts in different solvents.

Type of Extract	Percentage yield
Hot Methanolic extract (HME)	9.06%
Cold Methanolic extract (CME)	5.49%
Hot Hexane extract (HHE)	0.94%
Cold Hexane extract (CHE)	1.48%

Preliminary phytochemical analysis

The information obtained from preliminary phytochemical screening will be useful in finding out the genuity of the drug. Preliminary phytochemical analysis indicated the presence of carbohydrates, amino acids and flavonoids in methanolic extracts. *(Table3)*

Fluorescence Analysis of Powder sample

The behavior of drug powder with different chemical reagent will also be helpful in characterization of crude

Table 3: Preliminary phytochemical analysis of *Citrus aurantifolia* (Christm) Swingle peel extracts.

Test performed	Name of the test	HME	CME	HHE	CHE
Test for carbohydrates	Fehling's test	+ve	+ve	−ve	−ve
Test for Amino acids	Ninhydrin Test	+ve	+ve	−ve	−ve
Test for Flavonoids	Shinoda Test	+ve	+ve	−ve	−ve
Test for Alkaloids	Dragendroff Mayer's Wagner's reagent	−ve	−ve	−ve	−ve
Test for Tannins and Phenolic compound	5% FeCl3	−ve	−ve	−ve	−ve
Test for Steroids	Salkowski reaction	−ve	−ve	−ve	−ve

+ Denotes the presence of respective class of compound

Table 4: Fluorescence Analysis of dried peel powder of *Citrus aurantifolia* (Christm) Swingle.

Test Sample	Day Light	UV light (365nm)	UV light (254nm)
Powder	Pale Yellow	Whitish Yellowish	Pale Yellow
Powder+ D/W	Light Brownish Yellow	Light Brown	Light Yellow
Powder+1NNaOH aq.	Dark Yellow	Dark Yellow	Dark Yellow
Powder+1NNaOH alk	Light Lemon Yellow	Milky White	Dark Yellow
Powder+1N HCL	Pale Yellow	WhitishYellowish	Pale Yellow
Powder+ Conc.HCl	Dark Greenish Yellow	Dark Greenish Yellow	Greenish yellow
Powder+ Conc. H2SO4	Dark Reddish Brown	Dark Brownish Black	Brownish Black
Powder+Conc.HNO3	Brownish Yellow	Black	Greenish Yellow
Powder+ Chloroform	Brownish Yellow	Dark Brownish Yellow	Pale Yellow
Powder+ Acetone	Pale Yellow	Pale Yellow	Milky White
Powder+ Acetic acid	Pale Yellow	Milky White	Pale Yellow
Powder+Fecl3	Dark Brown	Black	Dark Brown

Table 5: Thin Layer Chromatography analysis of *Citrus aurantifolia* (Christm) Swingle peel extracts.

R_f Value	Color of the Band
0.2	Dark Purple
0.38	Dark Green
0.50	Dark Purple
0.55	Brown
0.60	Dark Blue
0.67	Green
0.74	Purple
0.87	Dark Blue

drug. The fluorescence characteristics of powdered drug were studied under U.V. light after treating with different chemical reagents and reported in *(Table4)*

Thin Layer Chromatography study

TLC is a simple, low-cost, versatile and specific method for the identification of herbal medicines.[9]The fingerprint has potential to determine authenticity and reliability of chemical constituents of herbal drug and formulations.[10] R_f values were calculated for methanolic and n-hexane extracts. *(Table 5) (Fig 4A, 4B, 4C, 4D.)*

Determination of Microbial Load

Medicinal plant materials normally carry a great number of bacteria and moulds, often originating in soil. While a large range of bacteria and fungi form the naturally occurring microflora of herbs, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, handling and production may cause additional contamination and microbial growth. The determination of microbial load may indicate the quality of production and harvesting practices. Total bacterial count was found to be *$2.2x10^2$ cfu/gm* which is less than the *WHO* limits. Absence of pathogens indicates that this powder sample can be use for formulation. *(Table 6)*

CONCLUSION

Standardization is essential measure for quality, purity and sample identification. Microscopic method is one of the simplest and cheapest methods to start with for establishing the correct identity of the source materials [11].The majority of the information on the identity, purity and quality of the plant material can be obtained from its macroscopy, microscopy, physio – chemical parameters and TLC fingerprinting. The present work is undertaken to obtain some pharmacognostical standards.The above studies provide information with respect to the identification, chemical constituents and physicochemical characters of *Citrus aurantifolia* (Christm) Swingle peel. These studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible

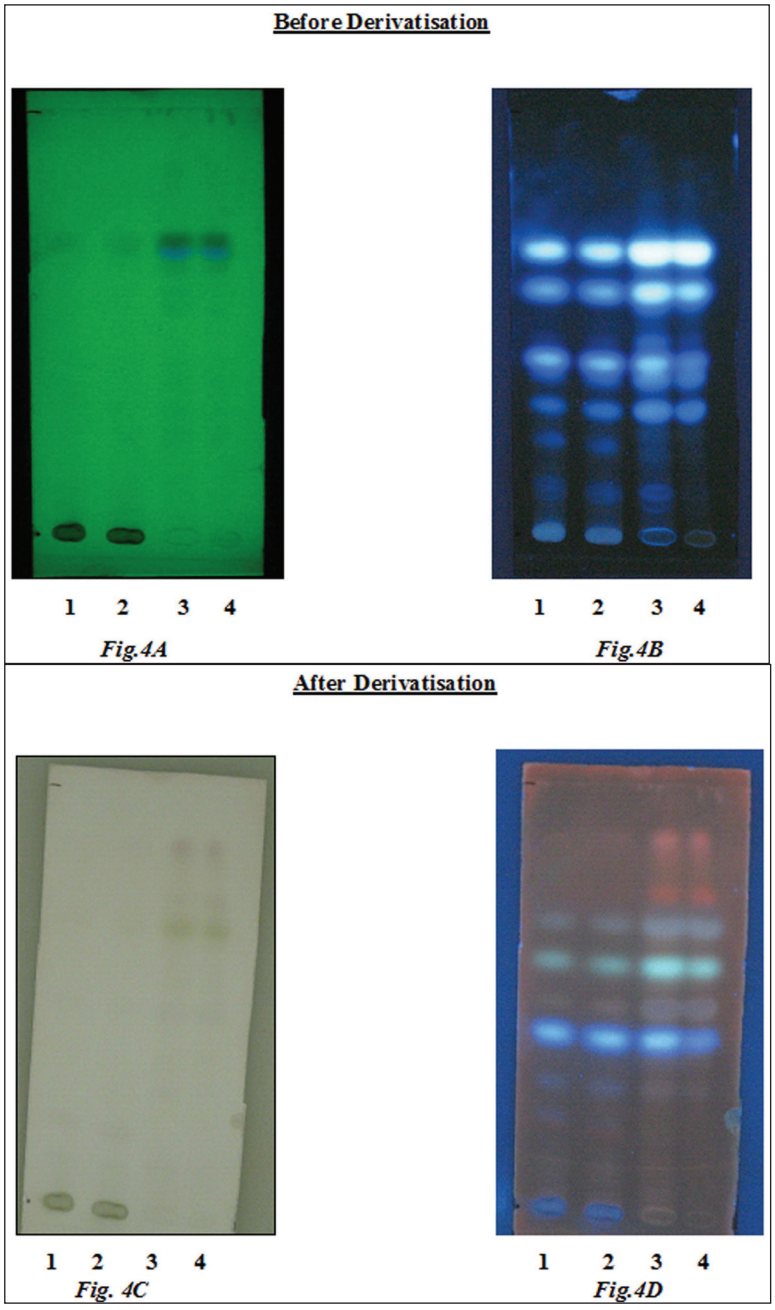


Figure 4A: TLC profile at 254nm before derivatisation
Figure 4B: TLC profile at 366nm before derivatisation
Figure 4C: TLC profile in visible light after derivatisation
Figure 4D: TLC profile at 366nm after derivatisation

Table 6: Determination of microbial load of dried peel powder of *Citrus aurantifolia* (Christm) Swingle.

Micrbial method & Pathogen	Microbial Count & Presence or absence of pathogen
Total bacterial count	2.2×10^2 *cfu/gm*
Yeast & Molds	Absent
E. coli	Absent
Salmonella	Absent
S. aureus	Absent

quality of herbal medicine which will contribute to its safety and efficacy. [12]

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Phyto-Pharmacognostical Studies of two endangered species of *Malaxis* (Jeevak and Rishibhak)

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ABSTRACT

Jeevak (*Malaxis muscifera* (Lindl.) Kuntze) and Rishibhak (*Malaxis acuminata* D.Don) belongs to family Orchidaceae, are critically endangered medicinal plants and two of the eight components of the vitality strengthening polyherbal Ayurvedic formulation "Astavarga". The botanical identity of both plants suffers a lot of confusion and doubts in Ayurvedic literature. In lack of proper production, supply system and the increasing demands of herbal drugs are the major factors promoting the practices of adulteration and substitution. Therefore the standardisation of herbal drugs are essential for assuring the therapeutic efficacy of drugs. The present study is to carry out and verify the botanical / pharmacognostical identity of genuine sample and their phytochemical differences of market samples used in the name of Jeevak and Rishibhak.

Key words: Macroscopy, Microscopy, Thin Layer Chromatography, physico chemical evaluation.

INTRODUCTION

Medicinal plants constitute an effective source of traditional and modern medicines. Standardisation of plant products is a complex task due to their heretogenous composition which is in the form of whole plant, plant parts or extracts obtained thereof. To ensure reproducible quality of herbal drugs, proper control of starting material is utmost essential[1].

The identity of two species Jeevak (*Malaxis muscifera* (Lindl.) Kuntze) and Rishibhak (*Malaxis acuminata* D.Don) of Astavarga groups belongs to family Orchidaceae (one of largest family of monocots[2] and largest among flowering plants), suffer a lot of confusion and doubts in Ayurvedic literature, till the introduction of taxonomic nomenclature. Jeevak and Rishibhak were first described for the first time in SodhalGuna and Samragaha, a medieval text of Ayurveda (ISM) followed by other texts like Bhavprakash Nighantu[3] (*Haritkyadivarga*), Dhanvantari Nighantu (Guduchayadivarga)[4] and were included as a component of *Jeevaniyagana* (in Charak Samhita[5]) and *Kakolyadigana* and *Padmakadigana* in Sushruta Samhita[6] and Astanghridaya[7] respectively.

Gradual decline in Natural habitat, rapid destruction of forests, climatic and ecological changes, all are responsible for severe loss of plant population and as a consequence these two species are becoming rare. Since there is no systematic study has been carried out so far, regarding the comparative account on market sample with respect to genuine sample for identification of raw drug. Therefore it was decided to take up all the drugs commonly supplied in the market, official substitutes and substitutes supplied in the drug market for Jeevak and Rishibhak, for detailed pharmacognostical and phytochemical studies, in order to establish the diagnostic features of both the species.

MATERIAL AND METHODS

Fresh tubers of botanically identified plants of *Malaxis muscifera* (Lindl.) Kuntze and *Malaxis acuminata* D.Don were collected from medicinal plants garden of Regional Research Institute of Himalayan Flora, Tarikhet and Indian Medicines Pharmaceutical Corporation Limited, Mohan (Almora). Tubers were washed, cut into pieces and

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preserved in Formalo-acetyl-alcohol (FAA) and labelled MM4 and MA4 for pharmacognostical study. Microtome section were taken, stained and mounted following the usual plant microtechniques[8,9] and representative diagram sketched through camera lucida and some were shade dried and coarse (20 to 30 #) powdered for qualitative tests and physico-chemical study as per WHO / API / IP guidelines. The physicochemical parameters like total ash, acid insoluble ash, water and alcohol soluble extractives of all the samples were carried out adopting standard procedures[10,11,12]. The thin layer chromatography of 90 per cent ethanolic extract of all samples were performed on pre-coated silica gel 60 F_{254} aluminium plates and the plates were developed in solvent system n-Hexane: Chloroform : Acetone: Acetic acid (6:3:1:0.5). The developed plates were observed under UV 254 nm and UV 366 nm and after derivatization visualization under UV 366 nm.

Market samples MM1 , MM2, MM3 and MA1, MA2, MA3 were collected in the name of Jeevak and Rishibhak from New Delhi, Jaipur (Rajasthan), Mandi (Himachal Pradesh), respectively and compared pharmacognostically as well as phytochemically with authentic drugs.

RESULTS

Macro and microscopical studies of *Malaxis muscifera* (Lindl.) Kuntze

Macroscopic

Dried pseudobulb conical in shape, straight or slightly curved, about 2 to 7 cm long and 0.5 to 1 cm thick, broader at the base with broken rootlets; surface dark brown to nearly black, wrinkled with 2 to 4 obliquely arranged rings of papery white remnants of dried leaf bases (Figure 1a); taste mucilaginous; odourless. Diagrammatic T.S. of transversely cut surface of the pseudobulb is circular to oval with irregularly crenate margins in out line and shows a layer of epidermis and wide parenchymatous ground tissue with scattered vascular bundles and mucilage cavities (Figure 1b).

Microscopic

Detailed transverse section of pseudobulb shows, a layer of epidermis of tubular, tangentially running cells of irregular size with thin cuticle followed by ground tissue of parenchymatous cells traversed by wide air spaces, vascular bundles, numerous mucilage cells and cells containing bundles of acicular crystals of calcium oxalate; vascular bundles consisting of 3 to 5 xylem vessels and a narrow zone of phloem (Figure 1c).

Powder

Light brown in colour. Shows abundant, oval or irregularly shaped parenchymatous and mucilage cells with acicular raphides; acicular crystals of calcium oxalate scattered singly or in broken pieces or in bundles scattered in parenchymatous cells and rarely reticulate vessels (Figure 1d).

Macro and microscopical studies of *Malaxis acuminata* D.Don

Macroscopic

Fresh pseudobulb conical in shape, fleshy, green, smooth, shining, 3 to 9 cm long and 1 to 3 cm broad, slightly mucilagenous, covered with shining, translucent light green (in fresh bulb) and light brown (in dry), membraneous, 3 or 4 sheathing leaves arranged alternately and having parallel venation; stem rudimentary and have a fibrous root; roots arises from the base. Whereas dried pseudobulbs conical, transluscent, reddish brown in colour, 2 to 5 cm long and 0.7 to 1 cm wide, surface rough, punctated, fracture hard; cut surface dark brown, coarsely granulated with irregular margins and white spots; pleasant smell; astringent, slightly mucilagenous in taste.

Microscopic

Transverse section of pseudobulb oval to circular in outline; section passing through scaly leaves which exfoliate, showing a single layered, thick walled, sclerified epiblema having acicular crystals of calcium oxalate, followed by mesophyll adjacent to the epiblema composed of 2 to 4 layers of elongated cells with lignified reticulate thickening (the lignification was confirmed with phloroglucinal and conc. HCl); devoid of chloroplast; vascular bundles prominent, phloem well developed with large sieve plates, surrounded by sclerenchymatous bundle sheath; section passing through bulb shows a layer of thick walled sclerified epiblema; with cuticle, which is also single layered, below this lie 1 or 2 layers of large sclerified cells and these extend unevenly into ground parenchymatous tissue; ground parenchyma irregular, with large air spaces with passage cells in the form of small protuberances at some places; parenchymatous cells towards epiblema were smaller in size while towards outer they are bigger; vascular bundles scattered throughout the ground tissue surrounded by thick walled sclerenchymatous cells, which occasionally extend into intercellular spaces; vascular elements showed scariform and spiral thickening (Figure 2a-2b).

Powder

Yellowish brown in colour, pleasant smell, slightly bitter and astringent in taste, shows groups of mesophyll cells

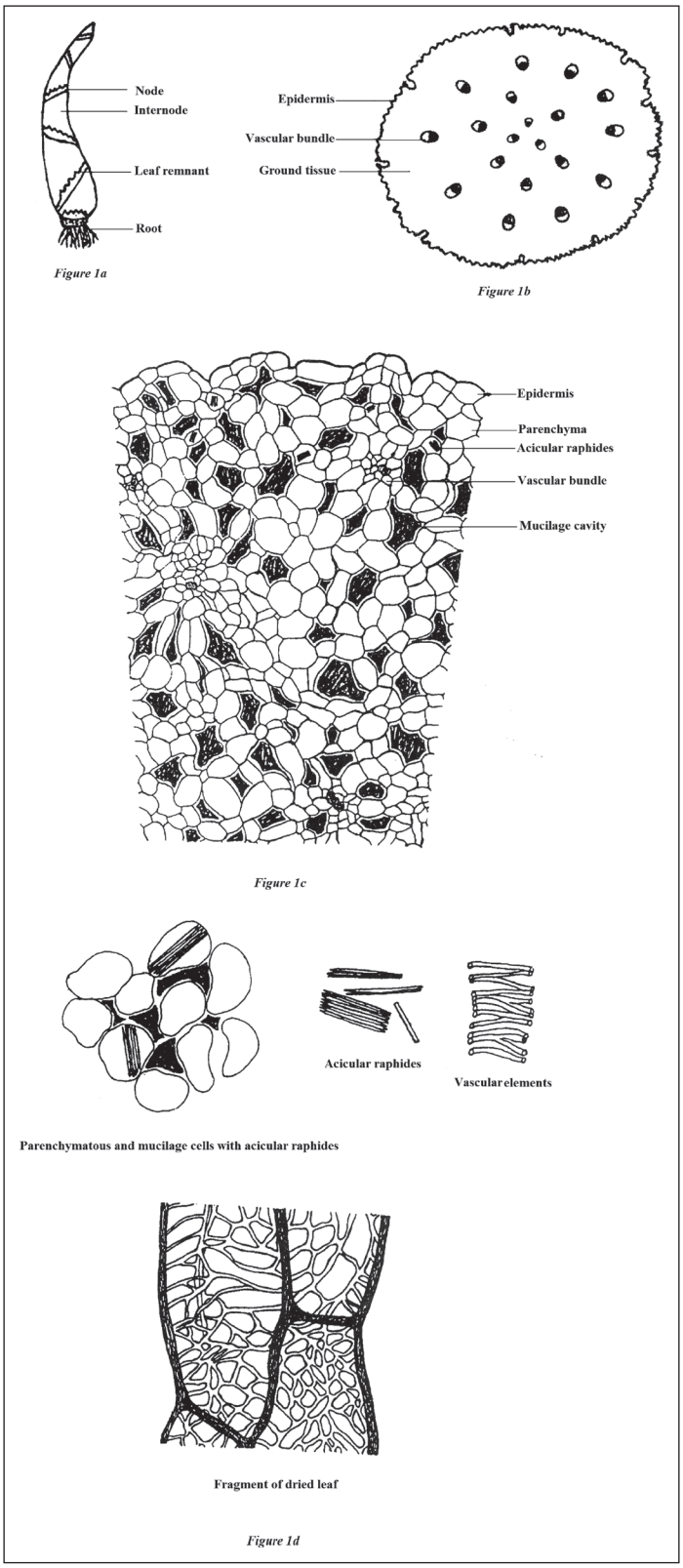

Figure 1a: Dried pseudobulb of *Malaxis Muscifera* (Lindl.) Kuntze
Figure 1b: T.S. of pseudobulb of *Malaxis Muscifera* (Lindl.) Kuntze (diagrammatic)
Figure 1c: T.S. of pseudobulb of *Malaxis Muscifera* (Lindl.) Kuntze (portion enlarged) (X40)
Figure 1d: Powder characteristics of *Malaxis Muscifera* (Lindl.) Kuntze

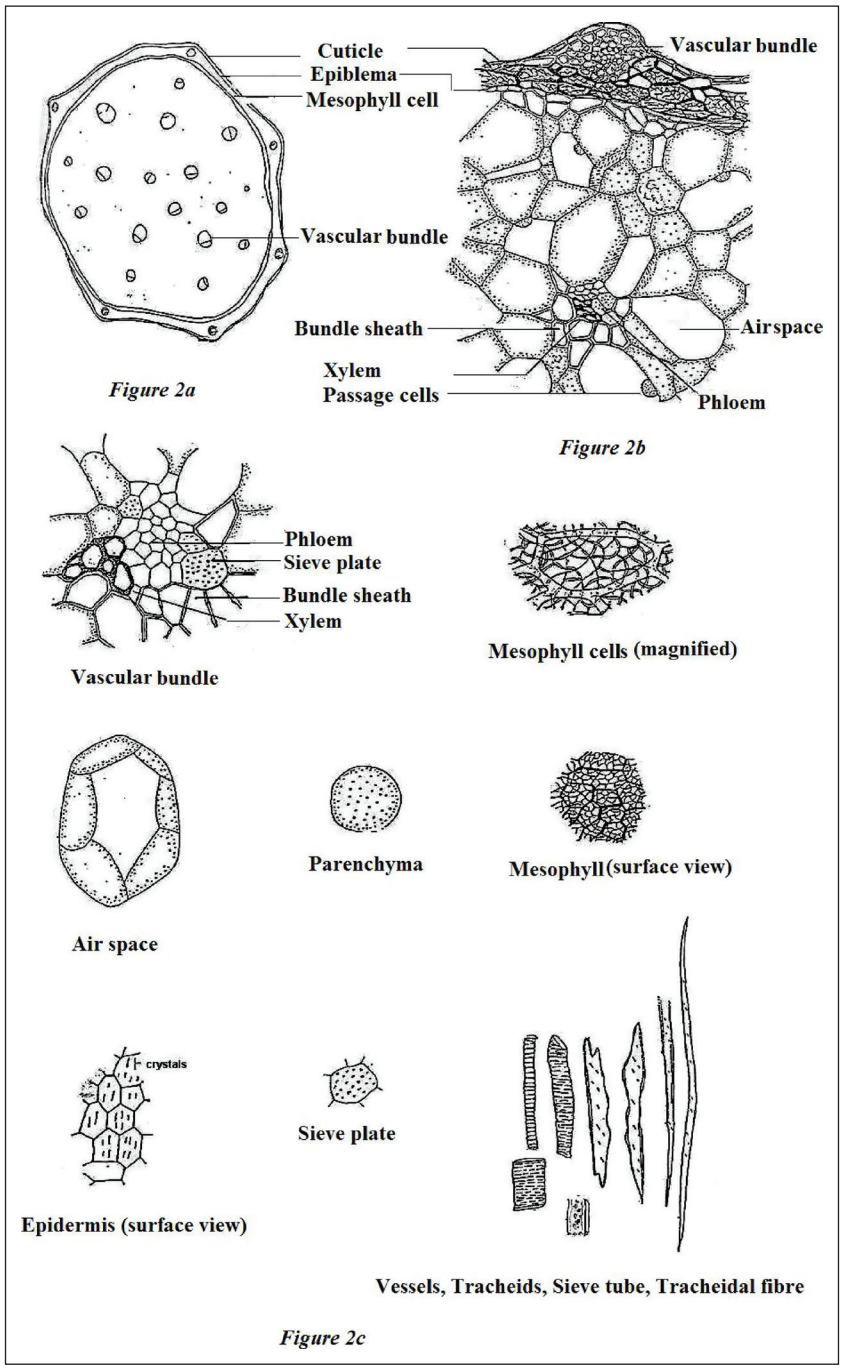

Figure 2a: T.S. of pseudobulb of *Malaxis acuminata* D.Don (diagrammatic)
Figure 2b: T.S. of pseudobulb of *Malaxis acuminata* D.Don (portion enlarged)
Figure 2c: Powder characteristics of *Malaxis acuminata* D.Don

with reticulate thickenings inside; vessels with spiral, scalariform and reticulate thickening; fibre tracheids of about 600 m long upto 80 m broad, and tracheids (about 19 m long and 40 m broad); groups of parenchyma with acicular crystals of calcium oxalate, sieve plates, sieve tubes and angular parenchymatous cells. Powder when treated with conc. HNO_3 on microscopic slide emits light green fluorescence under UV 365 nm (Figure 2c).

COMPARATIVE PHARMACOGNOSTIC CHARACTERS OF MARKET SAMPLES

The detail macro and microscopical characters of all the market samples MM1-MM3 and MA1-MA3 were studied and compared with *Malaxis* species and it was observed that MMI and MM3 shows more or less similar macro and microscopical characters with that of *M.muscifera* however

the characters of market sample MM2 resemble the pharmacognotic characters of *Pueraria tuberosa* DC., the dried tubers 3 to 5 cm long and 2 to 4 cm broad and fibrous; fresh tubers large, napiform, 30 to 60 cm thick, outer surface light brown in colour and cut surface creamy; fleshy, small transverse warts and ridges are found on the surface, a texture smooth, bitter and mucilaginous in taste, no particular odour (MM2). Transverse section of tuber is slightly wavy in outline, epidermis not distinct, 3 to 4 layers of cork cells followed by 5 to 7 layers of parenchymatous cells, cork cambium brown in colour and 2 to 3 celled thick. Endodermis well developed, pericycle fibres followed by two or three layers of stone cells filled with sandy crystals; groups of crystalloid fibres present in the phloem region. Scattered groups of xylem vessel elements seen. The medullary rays and phloem cells are filled with starch grains which are polygonal, 2-5 in diameter, simple or two to many compound, hilum usually indistinct, occasionally a central cleft, lamellae indistinct; xylem consists of vessel elements, tracheids, fibres and parenchyma; fibres multicellular articulated (Figure 5a-5c). Powder greyish brown, no characteristic odour, bitter in taste; shows parenchyma filled with starch, septate fibres in the form of crystals fibres as well as shaped bulb like pipette; vessels with simple and scalariform cross perforation plates, stone cells, and starch grains (Figure 5d).

The physico chemical analysis (Table 1) and TLC of 90 percent ethanolic extract (Figure 3,4) of powders of all the samples were carried out using solvent system Solvent System N-Hexane: Chloroform : Acetone: Acetic Acid (6:3:1:0.5) and observations were compared (Table 2).

The macro and microscopical characters of MA2 and MA3 are identified more or less same as *M.acuminata*. Sample MA1 resembles the macro and microscopical characters of *Ipomoea digitata* L. The root consists of thick pieces of different sizes, usually 2 to 8 mm in dia.; outer surface brownish and rough due to the presence of longitudinal fissures, ridges and numerous circular

Table 1: Observations of Physicochemical Parameters of Powdered Samples of Jeevak

S. No.	Parameters %		MM1	MM2	MM3	MM4
1.	Total Ash (% W/W)		6.5	3.6	6.2	5.0
2.	Acid Insoluble Ash (% W/W)		1.52	1.39	0.8	2.0
3.	Ethanol Soluble Extractive (% W/W)		5.0	7.0	7.5	6.0
4.	Water Soluble Extractive (% W/W)		11.0	13.0	5.18	10.0
5.	TLC (Figure 3)	Under UV 254 nm (R_f Values)	0.26, 0.35, 0.46, 0.89, 0.92	0.20, 0.59	0.23,0.68	0.26, 0.32, 0.41, 0.56, 0.86, 0.95
		Under UV 366 nm (R_f Values)	0.90	0.05, 0.08, 0.13, 0.19, 0.29, 0.39, 0.49, 0.53, 0.61, 0.66, 0.91	0.08, 0.11, 0.18, 0.23, 0.27, 0.38, 0.46, 0.52, 0.55, 0.87	0.06, 0.12, 0.26, 0.39, 0.48, 0.89, 0.94
		After derivatization visualization under UV 366 nm (R_f Values)	0.06, 0.018, 0.22, 0.30, 0.38, 0.44, 0.71	0.04, 0.26,0.30	0.0	0.11, 0.28, 0.38, 0.49, 0.59, 0.73

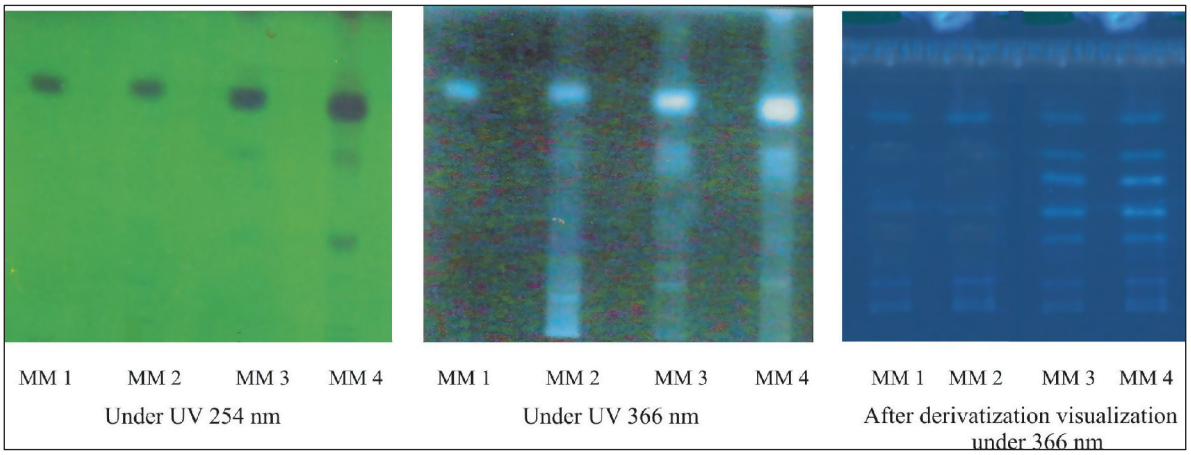

Figure 3: TLC fingerprint of 90% ethanolic extracts of samples

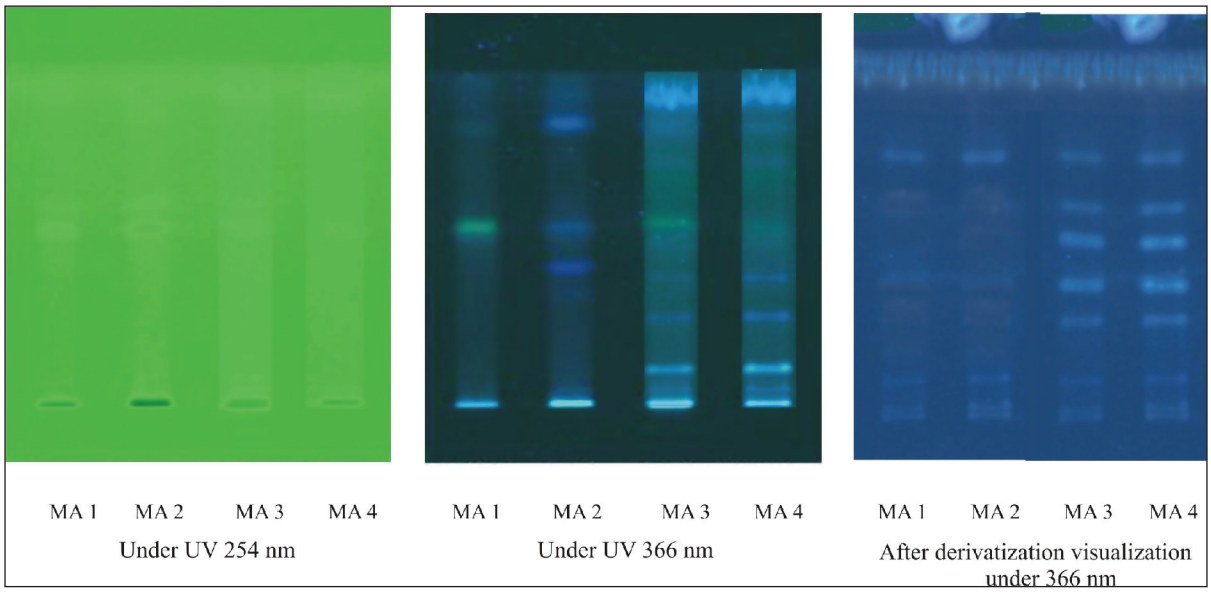

Figure 4: TLC fingerprint of 90% ethanolic extracts of samples

Table 2: Observations of Physicochemical Parameters of Powdered Samples of Rishibhak

S. No.	Parameters %		MA1	MA2	MA3	MA4
1.	Total Ash (% W/W)		4.2	5.3	5.9	6.2
2.	Acid Insoluble Ash (% W/W)		3.7	3.9	4.2	3.0
3.	Water soluble Ash (% W/W)		4.6	4.2	4.9	4.0
4.	Water soluble extractive (% W/W)		43.4	38.3	42.5	36.2
5.	TLC (Figure 4)	Under UV 254 nm (R_f Values)	0.04, 0.019, 0.05, 0.07	0.04, 0.07, 0.10	0.00, 0.01, 0.03, 0.09, 0.12	0.00, 0.01, 0.03, 0.09, 0.12
		Under Uv 366 nm (R_f Values)	0.11, 0.28, 0.38, 0.46	0.13, 0.31, 0.62, 0.82	0.05, 0.012, 0.29, 0.67, 0.95	0.05, 0.012, 0.29, 0.67, 0.95
		After derivatization visualization under UV 366 nm (R_f Values)	0.12, 0.23, 0.53, 0.64	0.15, 0.28, 0.59, 0.70	0.17, 0.38, 0.48, 0.59, 0.69, 0.83	0.17, 0.38, 0.48, 0.59, 0.69, 0.83

lenticels; core light brown and fibrous; fracture, fibrous, odourless and sweetish in taste. Transverse section of root (Figure 6a) shows 6 to 9 layers of thin walled cork cells, externally covered by rhytidoma; phelloderm composed of 8 to10 layers of cells, thin walled and filled with starch grains, individual starch grain rounded to irregular in shape, variable in size measuring about 13 to 24 µm, with distinct centric hilum; rosettes of calcium oxalate present; secondary phloem consists of companion cells, sieve tube elements and phloem parenchyma, traversed by uni- or biseriate medullary ray; numerous resin ducts and starch grains occur in the secondary phloem; secondary xylem consists of xylem parenchyma, xylem vessels, xylem fibres and tracheids; vessels large in size and numerous, light to dark brown, fine to coarse texture; simple and compound starch grains of variable size, crystals of calcium oxalate in prismatic and cluster form; pitted vessels; tracheids; parenchymatous cells with simple pits and long fibres with wide lumen and pointed ends (figure 6b).

DISCUSSIONS AND CONCLUSION

From the foregoing observations it has been seen that both the species of *Malaxis* viz., *M.muscifera* resemble to great extent in their morphological and histological characters like shape of pseudobulb, ground parenchymatous tissue with large air spaces and scattered vascular bundles in ground tissue. However both the species can be also distinguished from each other for example in *M.muscifera*, epidermal layer is made up of tubular tangentially running cells of irregular size, vascular bundles consisting of 2-4 xylem vessels and a narrow zone of phloem where as in *M.acuminata*, the

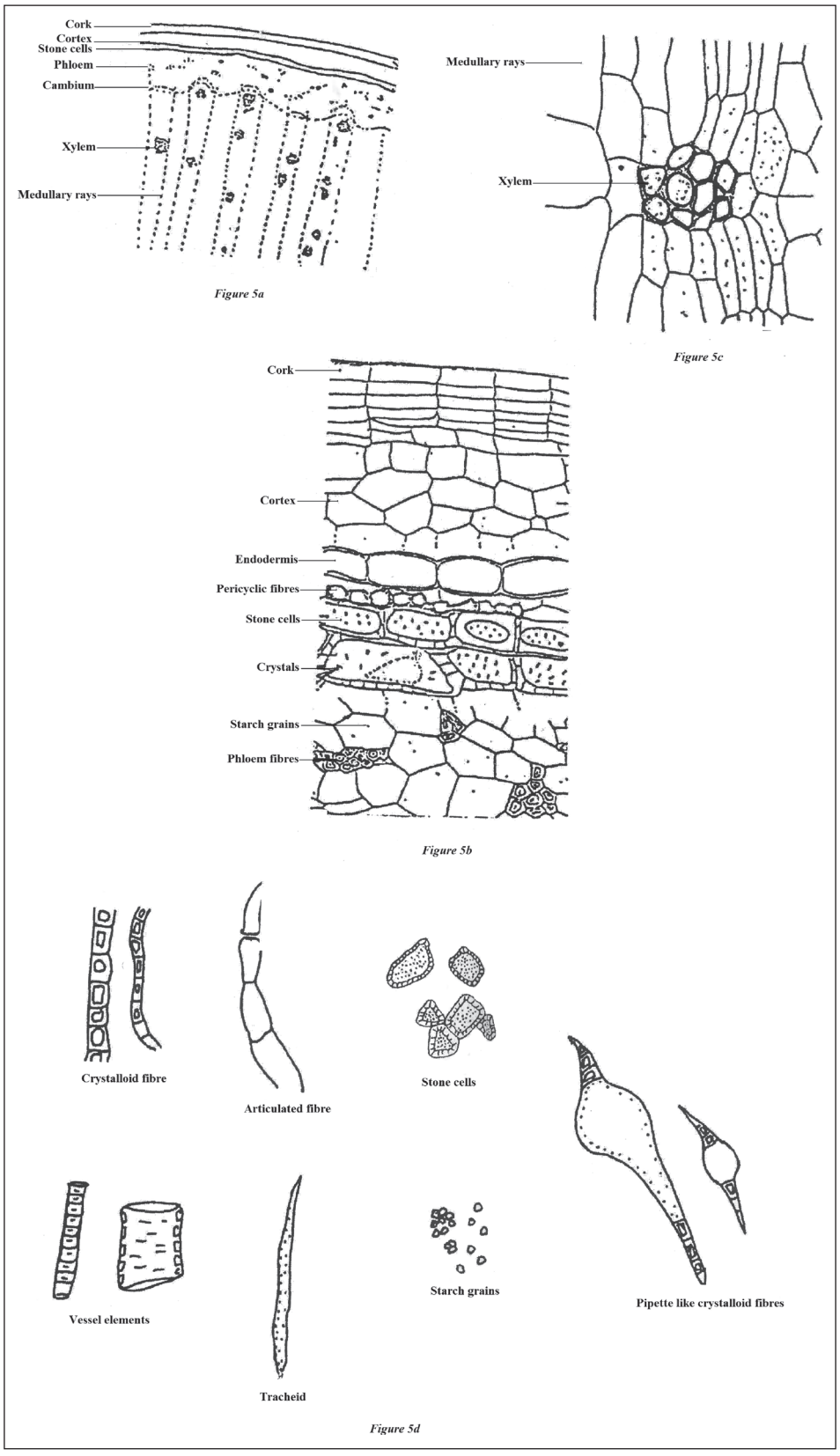

Figure 5a: T.S. of root tuber of *Pueraria tuberosa* DC. (diagrammatic)
Figure 5b: T.S. of root tuber of *Pueraria tuberosa* DC. (detail)
Figure 5c: T.S. of root tuber of *Pueraria tuberosa* DC. (xylem region)
Figure 5c: Powder characteristics of *Pueraria tuberosa* DC.

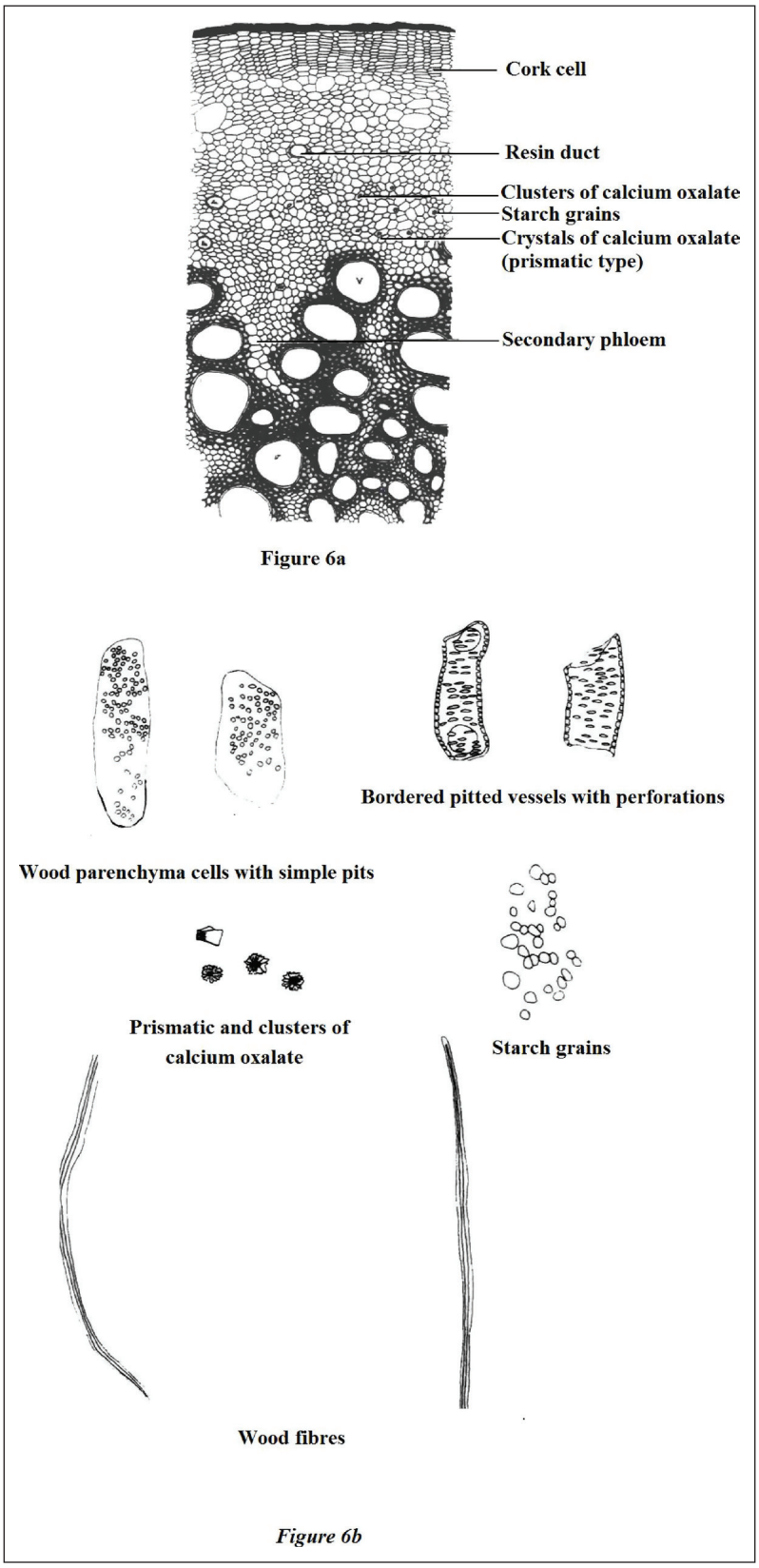

Figure 6a: T.S. of root tuber of *Ipomoea digitata* L. (Cellular details)
Figure 6b: Powder characteristics of *Ipomoea digitata* L.

epidermal layer is thick walled and sclerified and below this lie 1-2 layers of sclerified cells which extend evenly into ground tissue and vascular bundles are surrounded by thick-walled sclerenchymatous cells which occasionally extend into intercellular spaces. Similarly the anatomical structure of tuberous root of *Pueraria tuberosa* is quite characteristics and can be differentiated by having the epidermis not distinct, 3-4 layers of cork cells, cork cambium brown in colour, endodermis well developed, pericycle fibres followed by 2-3 layers of stone cells filled with sandy crystals. The medullary rays and phloem cells are filled with starch grains and the anatomical characters of *Ipomoea digitata* are quite characteristics by having 6 to 9 layers of thin walled cork cells, externally covered by rhytidoma; phelloderm composed of 8 to10 layers, thin walled cells, filled with starch grains, starch grains rounded to irregular in shape, variable in size with distinct centric hilum and rosettes of calcium oxalate crystals; secondary phloem consists of companion cells, sieve tube elements and phloem parenchyma, traversed by uni- or biseriate medullary ray; numerous resin ducts and starch grains occur in the secondary phloem; secondary xylem consists of xylem parenchyma, xylem vessels, xylem fibres and tracheids; vessels large in size and numerous.

The observation of physico-chemical evaluation indicates that the most drugs available in market are not genuine and adulterated with other plant drugs which are easily available. Adulteration of the genuine raw material is the main cause of degradation of desired therapeutic effect of plant species used in Ayurveda. Thus industries could utilize the scientific background for identification of raw material and this work is not only beneficial to the industries but also enhance the credibility of Indeginous System of Medicine.

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Hypoglycemic and *in Vitro* Antioxidant Effects of Methanolic extract of *Marsilea quadrifolia* Plant

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ABSTRACT

Introduction: Oxidative stress induced by alloxan has been shown to damage pancreatic beta cell and produce hyperglycemia in rats. Hence the present investigation has designed to appraise antidiabetic and antioxidant principle of the methanolic extract of *Marsilea quadrifolia* (MEMQ). **Methods:** Hypoglycemic effect was evaluated in alloxan induced diabetic rat. The oral administration of plant extract at a dose of 300 mg/kg body weight was given to fasting glucose loaded rat with regard to normal control during 1 hr. study period and in alloxan induced (110 mg/kg body weight i.p.) diabetic rat in comparison with reference drug Metformin Hydrochloride (100 mg/kg) during 3 days study period. The antioxidant potential of MEMQ was checked by qualitative method and quantitatively through DPPH(1,1-diphenyl-2-picryl-hydrazyl) scavenging assay at 517 nm. Total phenolic content, total antioxidant capacity and reducing power activity was also assayed. **Results:** Considerable drop in elevated blood glucose level was observed in the alloxan induced diabetic ($p<0.05$ & $p<0.001$) rat. At a dose of 300 mg/kg the extract showed glucose level reduction of 47.57% in alloxan induced rat while 44.38% was found for Metformin after 3 days. Antioxidant activity using DPPH was found to increase in a concentration dependent manner with an IC_{50} value of 96.37 ± 3.62µg/ml higher than the standard one, IC_{50} 16.59±0.59µg/ml. Total phenolic content was found 165.75 ± 0.961 mg/g in GAE and the total antioxidant capacity was equivalents of ascorbic acid (224.90 ± 1.42 mg/g). **Conclusions:** In all ways the extract showed significant antidiabetic and antioxidative potency. The present investigation suggests that MEMQ may be a potential source of natural antioxidant with good hypoglycemic activity.

Key words: *Marsilea quadrifolia*, Alloxan, Metformin Hydrocloride, Antioxidant activity.

INTRODUCTION

Free radicals cause depletion of immune system antioxidants, change in gene expression and induce abnormal proteins and contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, diabetes, cancer and AIDS[1].Oxidative stress may have significant effect in the glucose transport protein (GLUT) or at insulin receptor[2]. Scavengers of oxidative stress may have an effect in dropping the increased serum glucose level in diabetes and may lessen the diabetes as well as reduce its secondary complications. For this reason, chemotherapy including antioxidants finds increased use in the treatment of such diseases. This has stimulated the production of patented antioxidant preparations based on various components of natural or synthetic origin. The use of natural antioxidants for the treatment and prophylaxis of free radical induced pathologies has certain advantages. Most of these agents produce no side effects, possess low toxicity, and effectively act upon the main factors damaging the vascular system. Plants (fruits, vegetables, medicinal herbs) contain a wide variety of free radical scavenging molecules, such as phenolic compounds, nitrogen compounds, vitamins, terpenoids and some other endogenous metabolites, that are rich in antioxidant activity[3,4,5,6] . Diabetes is also the fourth-leading cause of death [7]. The diabetic population is rapidly increasing globally, particularly in the developing countries. South Asian region including Bangladesh is the most vulnerable focus. The current worldwide diabetic population is about 150 million and this will be doubled by 2025 [8]. The estimated prevalence of diabetes in Bangladesh is around 4%, which is similar to the average prevalence in many

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countries. But the prevalence of impaired glucose tolerance (IGT) here varies between 7.5-10% depending on urban and rural backgrounds [9]. A significant proportion of these patients obviously fall to get proper treatment and medication. Indigenous drugs since long, have been used for the treatment of diabetes [10]. Thousands of plants are known to have potential hypoglycemic effect. Bangladesh is abundant in antihyperglycemic plants. It is well established that diabetes is associated with low level of antioxidants and many plants show hypoglycemic property due to their antioxidant potential [11]. *Marsilea quadrifolia* (Marsileaceae), known as is an aquatic fern bearing 4 parted leaf resembling '4-leaf clover' (Trifolium). Leaves floating in deep water or erect in shallow water or on land. Leaflets obtained, to ¾" long, glaucous, petioles to 8" long; Sporocarp (ferns) ellipsoid, to 3/16 long, dark brown, on stalks to ¾" long, attached to base of petioles. A juice made from the leaves is diuretic and febrifuge and also used to treat snakebite and applied to abscesses etc.[12] . The plant is anti-inflammatory, diuretic, depurative, febrifuge and refrigerant[12-13] .The plant contains an enzyme named Thiaminase[13] . The Petroleum ether, chloroform and ethyl acetate extract showed antibacterial, cytotoxic and antioxidant activity [14]. In the present study was aimed to evaluate the effect of *Masilea quadrifolia* on blood sugar levels in alloxan treated rats and its antioxidant activity through various analytical methods.

MATERIALS AND METHODS

Collection and Identification of plants

The aerial parts of *Marsilea quadrifolia* were collected from Ramna, Dhaka, Bangladesh in July 2011 and were identified at the Bangladesh National Herbarium, Mirpur, Dhaka where the Voucher specimen no: 35441 has been deposited. The collected plant parts were separated from undesirable materials or plant parts. They were dried for one week. The plant parts were ground into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced.

Extraction of Plant Material

The collected aerial parts of plant was cleared, dried under shade at room temperature and powdered. For the extraction of phytochemicals about 200 gm powder of the plant was taken in a glass jar and completely submerged with 600 ml ethanol. The container with its content was sealed by cotton plug and aluminum foil and keep for a period of 7 days accompanying occasional shaking and stirring. The extract was then filtered through filter paper (Double Rings filter paper 102, 11.0 cm). The filtrates was concentrated at 50ºC under reduce pressure using vacuum pump rotary evaporator (STUART RF3022C, UK) to yield a solid residue 30gm.

Design of Experiment

The research work was carried out for about 4 weeks. In the first week various methods of *in-vitro* antioxidant screening methods and acute toxicity test were performed. And in the next 3 weeks diabetic condition was induced in the experimental rats and than treated with crude MEMQ for hypoglycemic activity study.

Experimental animal

Experiment was conducted on adult albino rats of either sex with the weights of 110-165 gm procured from International Centre for Diarrheal Disease Research Bangladesh (ICDDRB). All rats were fed normal laboratory chow food containing 16% protein, 66% carbohydrate, 8% fats and water. All rats were housed at a (12:12) hr light and dark cycle at 25°c and relative humidity (60-70) %.

Ethical Approval

The guidelines followed for animal experiment were accepted by the institutional animal ethical committee[15] .

Experimental Design

Animals were alienated into five groups and for every group six animals were taken.

Group I (Normal control) rats served as positive control received physiological saline (0.9% NaCl; 5ml/kg.b.w.p.o).

Group II (Diabetic Control) intraperitoneally injected Normal saline treated Alloxan induced Diabetic rat.

Group III rats were received intraperitonially administration of Metformin Hydrochloride (100 mg/kg/day) at a period of 24 hr for 3 successive days and served as standard.

Group IV rats were received *M. quadrifolia* (300mg/kg/day) orally at a hiatus of 24 hr for three consecutive days. Blood glucose was measured on 1st, 2nd and 3rd day.

Preparation of Alloxan solution

At first body weight of rats were measured. Then necessary amount of Alloxan was measured according to the body weight by following the dose of 110 mg of Alloxan per 1000 gm of body weight. Then calculated quantity of Alloxan was dissolved in 0.1 ml of sterile normal saline water.

Induction of alloxan

The rats were injected Alloxan monohydrate (Fluka, Germany), dissolved in sterile normal saline water at a dose of 110mg/kg body weights intraperitoneally once a day. Alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin discharge; therefore the rats were treated with glucose solution orally. After few days rats with moderate diabetes having glycosuria and hyperglycemia that is blood glucose level go beyond normal level were chosen.

Preparation of dosage of active drug and plant extract

Metformin hydrochloride (a biguanide): Metformin hydrochloride was in microcrystalline form and freely soluble in water. The dosage was prepared in solution form with sterilized water in such a concentration that each 0.1ml contained metformin hydrochloride according to the dose of 100 mg/kg/day, seeing as metformin is effective in such dose in case of humans.

***M. quadrifolia*:** The crude extract obtained and was dissolved in Tween 80 and water to prepare the solution where each 1 ml contained *M. quadrifolia* according to the dose of 300 mg/kg/day. 1 ml of the tested solution was administered everyday during treatment to achieve required dose of respective agents.

Hypoglycemic activity

Glucose tolerance test

A glucose tolerance test is the administration of glucose to determine how quickly it is cleared from the blood. The rat were tested in a fasting state (having no food or drink except water for at least 10 hours but not greater than 16 hours). An initial blood sugar was drawn and then the rat were fed glucose. The rat then had their blood tested again 30 minutes, 1 hour, 2 hours and 3 hours after drinking the high glucose drink [16].

Blood sugar assessment

Fasting blood glucose level was evaluated in normal and diabetic rats from the tail vein by strip technique (Bioland Glucometer, Germany). At first it is done just prior to extract administration of first day then it is continued for 3 days just one hour after the administration of plant extract.

In vitro antioxidant activity screening

***Qualitative assay*:** A suitable diluted stock solution was spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted [17].

Quantitative assay

The amount of phenolic compounds

Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be anticipated colorimetrically at 650 nm. The total phenolic content of MEMQ was determined using Folin-ciocalteu reagent[18] method. The content of total phenolics in the MEMQ was calculated from regression equation of the calibration curve ($y = 0.0138x+0.1275$, $R^2 = 0.9881$) and is expressed as galic acid equivalents (GAE).

Determination of total antioxidant capacity

The antioxidant activity of the MEMQ was evaluated by the phosphomolybdenum method according to the procedure of Prieto [19]. The antioxidant activity is expressed as the number of equivalents of ascorbic acid using the following equation:

$$C = (cxV)/m$$

Where, C = total antioxidant activity of plant extract in Ascorbic acid (mg/ml), c = Concentration of Ascorbic acid established from the calibration curve (mg/ml), V = volume of extract (ml), m = weight of pure plant extract(g).

Free radical scavenging activity measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH)

Based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical, the free radical scavenging activity of MEMQ extract, was determined by the method described by Braca [20]. The percentage inhibition activity was calculated from:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract/ standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration ($\mu g\ mL^{-1}$) versus % inhibition.

Reducing power assay

The reducing power of MEMQ was determined according to the method described by Oyaizu [21]. Increased absorbance of the reaction mixture indicated increased reducing power.

Table 1: Glucose tolerance test after administration of 100mg/kg & 200 mg/kg body weight of extarct on rat.

Group	Blood glucose level			
	Initial (mmol/L)	1 h (mmol/L)	2 h (mmol/L)	3h (mmol/L)
Normal control	4.0 ± 0.152	3.45 ± 0.400	2.89 ± 0.020	2.40 ± 0.140*
Diabetic extract	4.65 ± 0.30	5.55 ± 0.129*	3.90 ± 0.119	2.98 ± 0.182
MEMQ (100 mg/kg)				
Diabetic extract	4.34 ±	5.75 ± 0.323	3.23 ± 0.327	2.67 ± 0.54*
MEMQ (200 mg/kg)	0.647			

Values are mean±SEM, (n=6);*: p<0.05, Dunnett's test as compared to control.

Table 2: The effect of three days treatment of methanolic extract of *M. quadrifolia* on blood sugar level of alloxan induced diabetic rat.

Group	1st day	2nd day	3rd day
Normal control	5.10 ± 0.17	5.02 ± 0.400	5.4 ± 0.320
Diabetic control	19.11 ± 0.90*	19.117 ± 2.029*	19.115 ± 2.019*
Metformin treated	12.46 ± 0. 67**	8.75 ± 0.31**	5.53 ± 0.27**
MEMQ (300 mg/kg/day)	14.38 ± 0.83**	9.83 ± 0.66**	6.83 ± 0.29**

Values are mean±SEM, (n=6);*: p<0.05, **: p< 0.001, Dunnett's test as compared to control. An experimental group IV rat at the dose of 300 mg-1kg was compared with diabetic control group II on corresponding day using Dunnett's test.

Table 3: Total amount of plant phenolic compounds and total antioxidant capacity of methanolic extract of *M. quadrifolia*

Sample	Total Phenols mg/g plant extract (in GAE)[a]	Total antioxidant capacity mg/g plant extract (in ASC)[b]
MEMQ	165.75 ± 0.961	224.90 ± 1.42

[a] Gallic acid equivalents (GAE, mg/g of extract) for the total phenolic content, [b] Ascorbic acid equivalents (mg/g of extract, mg/g of extract). The GAE and ASC values are expressed as Means±SEM of triplicate experiments.

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by the analysis of variance (ANOVA) follow Dunnet's multiple comparisons test, using SPSS 15.00 (USA) and Graph pad prism-5. For all the tests, results with *P* values < 0.05 & < 0.001 were taken to imply statistical significance.

RESULTS

Antidiabetic investigation

The result of glucose tolerance test and antidiabetic study are summarized in Table 1 and Table 2.

In vitro antioxidant activity

Qualitative assay

The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands.

Quantitative assay

Total Phenolic content

Total phenolic content of extract of *M. quadrifolia* was found 165.75 ± 0.961 mg/g in GAE and the result is shown in Table 3.

Total antioxidant capacity

Table 3 showed Total antioxidant capacity of methanolic extract of *M. quadrifolia* as the number of equivalents of ascorbic acid (224.90 ± 1.42 mg/g).

DPPH radical scavenging assay

The percentage (%) scavenging of DPPH radical was found to be concentration dependent. The results of DPPH scavenging activity with IC_{50} value of the experimental extract and the standard ascorbic acid are given in Table 4.

Reducing power assay

For the measurement of the reductive capacity, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of extract. Like the antioxidant activity, the reducing power of *M. quadrifolia* amplified with increasing concentration of the sample. Figure 1 exhibits the reductive ability of MEMQ compared with ascorbic acid.

Acute toxicity Test

The extract was safe up to a dose of 1600 mg/kg body weight. Behavior of the animals was closely observed for

Table 4: DPPH scavenging power of free radical of MEMQ and ascorbic acid

Sample	DPPH method IC_{50} (µg/ml)
MEMQ	96.37 ± 3.62
Ascorbic acid	16.59 ± 0.59

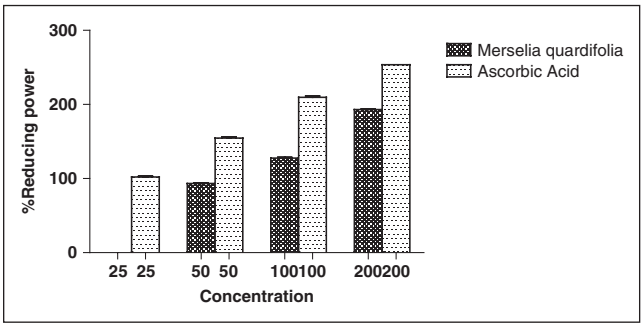

Figure 1: Reducing power of MEMQ and Ascorbic acid by spectophotometric detection of Fe^{3+} to Fe^{2+} transformation.

the first 3 h then at an interval of every 4h during the next 48 h. the extract did not cause mortality on rats during 48h observation or any behavioral change.

DISCUSSION

Hypoglycemic activity

Present study indicates that MEMQ (300mg/Kg b. wt) significantly decreased serum glucose level in hyperglycemic rats. Alloxan is the most frequently employed agent for the induction of experimental diabetic animal models of human insulin-dependent diabetes mellitus. There is escalating evidence that alloxan caused diabetes by rapid exhaustion of a cells, by DNA alkylation and gathering of cytotoxic free radicals that is suggested to result from initial islet inflammation, followed by infiltration of activated macrophages and lymphocyte in the inflammatory focus. It leads to a fall in insulin release there by a drastic diminution in plasma insulin concentration leading to stable hyperglycemic states[22]. It induces diabetes by dose dependent destruction of β-cells of islets of langrhans[23-24] . So, in the present study alloxan was chosen to create diabetic condition in rat and significant hyperglycemia was achieved within 48 hours after alloxan (110g/kg b.w. i.p) injection. The results obtained (Table1) showed that after a single administration of glucose 200 mg/kg in rat, there was a significant reduction ($p < 0.05$) of fasting blood glucose level during the 3 h study period. The research on Antidiabetic activity in alloxanised rats, administration of MEMQ of 300mg/kg body weight administered for 3 days was able to correct this anomaly significantly ($p<0.05$ & $p<0.001$). Significant reduction of blood glucose was observed from the 3rd day of the study. The comparable effect of the experimental extract with Metformin HCl may suggest similar mode of action since alloxan permanently destroys the pancreatic ß cells and the extract lowered blood sugar level in alloxanised rats, indicating that the extract possesses extra pancreatic effect. On the progression of treatment with methanolic extract of *M. quadrifolia* (300 mg/kg body weight) produced maximum reduction to 6.83±0.29 mmol/L on 3rd day whereas reduction to 5.53±0.27 mmol/L was found for metformin on 3rd day (Table 2). These observations suggest that the experimental extract might acquire insulin like effect on peripheral tissues either by promoting glucose consumption metabolism or inhibiting hepatic gluconeogenesis since alloxan treatment causes permanent destruction of ß cells [25].

In vitro antioxidant activity

Total phenol content

Phenolic compounds are commonly found in both edible and nonedible plants and they have been reported to have copious biological effects, including antioxidant activity. The antioxidant property of phenolic compounds is due to their redox properties, which can play a vital role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [26]. In this current study MEMQ possessed phenolic compound 165.75 ± 0.961 mg/gin GAE.

Total antioxidant capacity

Total antioxidant capacity of the crude extract, expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The result revealed the reducing power of the crude extract. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [27]. Many plants contain substantial amounts of antioxidants including Vitamin C and E, carotenoids, flavonoids, tannins and thus can be utilized to scavenge the excess free radicals from the human body[28] .

DPPH radical scavenging activity

DPPH is relatively stable nitrogen centred free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. This activity was increased by increasing the concentration of the sample extract. DPPH antioxidant assay is based on the ability of 1,

1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. From the results, it may be postulated that the plant extracts have hydrogen donors thus scavenging the free radical DPPH. Based on the data obtained from this experiment, DPPH radical scavenging activity of MEMQ (IC_{50} 96.37±3.62µg/ml)was drastically higher than the standard one (IC_{50} 16.59±0.59µg/ml).

Reducing power assay

The reducing capacity of a compound may furnish as a valuable indicator of its potential antioxidant power [29].At the concentrations the extract tested the reducing power of the investigated plant was increasing along with the standard ascorbic acid. The present data on the reducing power of the studied plant extract recommended that it is likely to contribute the antioxidant activity.

Acute toxicity Test

The absence of mortality and signs of toxicity up to 5 times the maximum effective dose, with MEMQ proves for that the plant has wide safety margin.

CONCLUSION

Our results support the view that some herbal medicinal plants did not inhibit glucose diffusion using in vitro model glucose absorption. In particular, their phenolic compounds and antioxidant activities may be useful for meal planning in type2 diabetes. They could contribute to sustain plasma antioxidant level because antioxidants present in the plants and herbs prevent the development of vascular diseases seen in type 2 diabetes. The preliminary investigation on the antidiabetic efficacy of MEMQ will be significant to proceed further in this path for the isolation of active principles responsible for antidiabetic activity. The antioxidant activities of medicinal plants may be due to the occurrence of phenolic compounds, containing the hydroxyl groups that confer the hydrogen donating ability. The present inquiry suggests that medicinal plants which acquire good antioxidant potential are the best supplements for the diseases coupled with oxidative stress. But we still don't know which chemical components are exactly responsible with the aforementioned effects so to find out the lead compounds liable for aforesaid activities from the above plant are in progress.

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