Phytochemical and Antimicrobial analysis of the crude extract, petroleum ether and chloroform fractions of Euphorbia heterophylla Linn Whole Plant

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ABSTRACT

The whole plant of *Euphorbia heterophylla* Linn family Euphorbiaceae, was screened for secondary metabolites and evaluated for its antimicrobial activities using standard procedures. The antibacterial activities of the methanol extract, pet ether, chloroform and methanol: water fractions were tested on gram negative and gram positive bacteria (*Staphylococcus albus*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumonae*) while the antifungal activity of the extract and fractions were tested on *Fusarium oxysporum, Aspergillus flavus*, *Aspergillus niger* and *Candida albicans*. The phytochemical screening showed the presence of alkaloids, tannins, cardiac glycosides and saponins in the whole plant of *E. heterophylla*. The active constituents present in the whole plant of *E. heterophylla* against the test organisms are of varying polarities with the crude extract showing significant activity against *Staph albus*, *Proteus mirabilis*, *E. coli*, *Salmonella typhi* and *Kleb. pneumonae* at all the doses tested and no effect on *Staph. aureus, Fusarium oxysporum, Aspergillus flavus and Candida albicans.*

Key word: *Euphorbia heterophylla* Linn, antimicrobial, pet. ether, chloroform fractions

INTRODUCTION

Infectious diseases are main causes of morbidity and mortality in man, especially in developing countries.[1] The emergence of multidrug resistant organisms to known antibiotics leading to several deaths worldwide has led to the interest of scientists in exploring plants, herbal products and traditional medicine for the identification of safe and effective remedies to ailments of microbial origin.[2]

Euphorbia heterophylla Linn belongs to the family Euphorbiaceae, a family represented by the trees, shrubs, herbs and characterized by presence of white milky latex which is more or less toxic.[3] It consists of about 300 genera and about 7,000 species.[4] The genus *Euphorbia* is one of the largest genera in the Euphorbiaceae family with about 1,600 species[5] which has been subjected to numerous chemical studies.[6] *E. heterophylla* Linn (syn. *E. geniculata*) commonly known as spurge weed is an erect, annual weed growing to about 3 ft high and locally abundant.

In East Africa, *E. heterophylla* is used for the treatment of gonorrhea and to accelerate wound healing. It is also used as a purgative,[7] a lactogenic agent,[8] as a cure for migraine and warts[9,10] while, the latex of the plant is used as fish poison, insecticide and poisons.[11] Previous biological studies have reported the antibacterial activity of the leaf of *E. heterophylla*,[9,12] its anti-inflammatory activity[13] as well as the wound healing potentials.[14]

Several compounds such as friedelin, β-sitosterol, myricyl alcohol, ellagic acid were isolated from the stem of *E. heterophylla*. Alanine, cysteine, serine, aspartic acid, methionine, proline, glutamic acid, stigmasterol, lupeol, beta-amyrin, stigmasterol glucoside, benzoic acid, 4-hydroxylbenzoic acid and quercetin were isolated from the leaves.[10,15] In addition, the roots have been reported to possess diterpenes.[16]

This study attempts to investigate the antibacterial and antifungal activities of the ethanol crude extract, pet ether and chloroform fractions of the whole plant of *E. heterophylla*.

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MATERIALS AND METHODS

Plant collection and authentication

Whole plant of *E. heterophylla* was collected at Sagamu, Ogun State, Nigeria and authenticated at the Forestry Research Institute of Nigeria FRIN with voucher number F.H.I. 100463 were a voucher specimen was deposited.

Plant preparation and extraction

The air dried and powdered whole plant of *E. heterophylla* was refluxed with ethanol for eight hours. The crude extract was evaporated to dryness to give the ethanol extract. The dried crude ethanol extract was reconstituted in methanol-water (1:3) and partitioned successively against pet ether and chloroform.

Phytochemical Screening.

The air dried powdered plant was screened for the presence of secondary metabolites by using standard procedures.[17]

Microorganisms

Laboratory culture of gram positive and gram negative bacteria (*Staphylococcus aureus*, *Staphyloccocus albus*, *Proteus mirabilis*, *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumonae)* were obtained from the microbiology Department of the Faculty of Basic Medical Sciences, Olabisi Onabanjo University while the fungi were obtained from the laboratory stock of the Department of Pharmaceutical Microbiology, University of Ibadan.

Preparation of bacterial cultures

Each organism was taken from prepared agar slope and inoculated into each of the six 5 mls of nutrient broth. The broth culture was incubated at 37°C for 24 hours. 0.1 ml of 1 in 100 dilution of the overnight culture was added to 20 mls of the melted and cooled nutrient agar at 45°C. The bottles were rolled between the palms to mix and poured into labelled sterile Petri dishes. The seeded Petri dishes were allowed to sit and dried in an incubator for twenty minutes by means of a sterile cork borer (diameter 0.7 cm).

The concentration of the crude extract, pet ether and chloroform fractions (90 mg/ml; 45 mg/ml and 22.5 mg/ml). The antibiotic discs used were Ampicillin, Gentamycin, Nitrofurantoin, Tetracycline and Colistin.

The plates were left at room temperature for six hours to allow the plant extracts diffuse into the agar medium and then incubated at 37°C for twenty-four hours.

Preparation of fungal cultures

Fungal organisms except *Candida albicans* were inoculated in malt extract broth. These were incubated at room temperature for four days and 0.5 ml of each was introduced into 30 mls saline to make 1 in 60 dilutions. 0.3 ml of each dilution was spread over the surface of the Petri dishes containing the Tryptone soya agar. Tryptone soya agar was prepared by dissolving 1 g of Tryptone Soya agar in 250 mls of water and boiled, the agar was then dispensed in 15 mls into universal bottles and autoclaved at 121°C. Cork borer (diameter 0.7 cm) was used to bore wells. 90 mg/ml; 45 mg/ml and 22.5 mg/ml concentrations of *E. heterophylla* whole plant extract were used and Tioconazole (5 µg/ml) was used as control. The plates were left at room temperature for six hours to allow the plant extract diffuse into the agar medium and then incubated at 37°C for ninety-six hours.

RESULTS

Results and Discussion

Previous phytochemical analysis have reported the absence of alkaloids,[13,15] Tannins,[15] saponins[18] and cardiac glycosides in the leaves of *E. heterophylla*[1,15] however, this study reports the presence of alkaloids, tannins which conformed with some previous reports,[18,19] cardiac glycosides[18] and saponins in the whole plant of *E. heterophylla.*

Table 1: Phytochemical screening analysis of *E. heterophylla* whole plant

Alkaloids	Cardiac glycoside	Saponin glycosides	Anthraquinone	Cyanogenetic glycosides	Tannins
+	+	+	–	–	+

– = Absent, + = Present.

Table 2: Antimicrobial screening of the crude extract of *E. heterophylla* whole plant

Dose	ZONE OF INHIBITION (cm)					
	E. coli	*Staph. aureus*	*Proteus mirabilis*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Staph albus*
90 mg/ml	1.8	0.0	1.1	1.5	1.4	1.8
45 mg/ml	1.4	0.0	1.0	1.0	1.3	1.7
22.5 mg/ml	0.0	0.0	0.6	0.4	1.0	1.4

Table 3: Antimicrobial screening of the pet ether fraction of *E. heterophylla* whole plant

Dose	ZONE OF INHIBITION (cm)					
	E. coli	*Staph aureus*	*Proteus mirabilis*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Staph albus*
90mg/ml	1.1	0.0	0.0	1.1	1.3	1.5
45 mg/ml	0.8	0.0	0.0	0.9	1.3	1.3
22.5 mg/ml	0.0	0.0	0.0	0.7	1.2	1.1

Table 4: Antimicrobial screening of the chloroform fraction of *E. heterophylla* whole plant

Dose	ZONE OF INHIBITION (cm)					
	E. coli	*Staph. aureus*	*Proteus mirabilis*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Staph. albus*
90 mg/ml	0.0	0.0	0.0	1.5	0.0	0.8
45 mg/ml	0.0	0.0	0.0	0.8	0.0	0.0
22.5 mg/ml	0.0	0.0	0.0	0.6	0.0	0.0

Table 5: Antimicrobial screening of the MeOH – H_2O of *E. heterophylla* whole plant

Dose	ZONE OF INHIBITION (cm)					
	E coli	*Staph aureus*	*Proteus mirabilis*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Staph. albus*
90 mg/ml	1.4	0.0	0.0	0.0	0.0	1.4
45 mg/ml	0.0	0.0	0.0	0.0	0.0	1.3
22.5 mg/ml	0.0	0.0	0.0	0.0	0.0	0.0

Gentamycin(10 mcg/ml) = 2.1 cm (Staph albus); Ampicillin (10mcg)= 1.1 cm (Proteus mirabilis); Tetracycline (10 mcg) = 0.6 cm( ); Nitrofurantoin (200 mcg) = 1.4 cm (Kleb pneumonia); Nalidixic (30 mcg) = 2.0 cm (E. coli); Chloramphenicol (10 mcg) = 0.4cm (Salmonella typhi)
(Diameter of cork borer = 0.7 cm).

Table 6: Antifungal screening of the MeOH – H_2O of *E. heterophylla*

Dose	ZONE OF INHIBITION (cm)			
	Fusarium oxysporium	*Aspergillus niger*	*Aspergillus flavus*	*Candida albicans*
90 mg/ml	0.0	2.4	0.0	0.0
45 mg/ml	0.0	0.0	0.0	0.0
22.5 mg/ml	0.0	0.0	0.0	0.0

Tioconazole (5 µg/ml) *A. flavus*; *A. niger*, *Candida albicans* = 2.0 cm.
(Diameter of cork borer = 0.7 cm).

The presence of saponin and cardiac glycosides in *E. heterophylla* showed that the diverse groups of compounds in the plant are related biosynthetically. The crude extract of the whole plant of E. *heterophylla* showed significant antibacterial activity against *Staph albus*, *Proteus mirabilis*, *E. coli*, *Salmonella typhi* and *Kleb. pneumonae* at all the doses tested and had no effect on *Staph. aureus*. The biological activity observed in the crude extract was however retained in the pet ether fraction for *E. coli, Salmonella typhi, Klebsiella pneumonia* and *Staph. aureus* while the chloroform fraction had significant activity against Salmonella *typhi which* showed the active constituents are of varying polarities.

There was no activity observed in the crude extract, pet ether and chloroform fractions of *E. heterophylla against Staph. aureus* while the activity observed for *Proteus mirabilis* in the crude extract was lost in both the pet. ether and chloroform fractions.

Against *Klebsiella pneumonae*, the crude extract activity was replicated in the pet ether fraction suggesting that the active constituents are non-polar while for *Staph albus*; the activity of *E. heterophylla* was more in the non-polar fraction and in the mother liquor again showing that the active constituents are of varying polarities.

The extract of *E. heterophylla* had comparable activity as Nitrofurantoin on *Klebsiella pneumonae* while it had a slightly lower activity compared to Nalidix acid and Gentamycin on *E. coli* and *Staph. albus*. The activity of *E. heterophylla* was comparable to that of Ampicillin on *Proteus mirabilis* and that of Colistin on *Salmonella typhi*.

E. heterophylla exhibited antifungal activity against *A. niger* and no activity on *Fusarium oxysporum*, *A. flavus* and *Candida albicans*. The presence of saponins and alkaloids has been reported to be responsible for various pharmacological properties with alkaloids exerting toxic effects against cells of foreign organisms.[20,21] The broad spectrum antimicrobial activity exhibited by the ethanol extract, pet. ether and chloroform fractions of *E. heterophylla* could therefore be

attributed to the presence of the alkaloid, cardiac glycosides, tannins, saponins in the whole plant of *E. heterophylla*. Further study is required to identify the active constituents responsible for these antibacterial and antifungal activities.

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Safety Assemment of *Centella asiatica* in albino rats

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ABSTRACT

Aim: Present study was designed to assess the safety levels of *Centella asiatica* (aerial pars) after 30 days oral administration in albino rats. **Materials & Methods:** Control group (I) received distilled water and test groups (II, III and IV) received graded dosage of 250, 500, 1,000 mg/kg. b.wt orally respectively for a period of 30 days. Changes in body weight were recorded at 10 days interval. On 32nd day blood samples were collected and the whole blood was used for hematological studies, DNA fragmentation assay and marker enzymes levels were assessed in serum. The vital tissues (Liver, Kidney, Heart, Spleen, and Brain) were dissected out and utilized for viability assay (Trypan blue dye exclusion test), evaluation of apoptosis (fluorescence microscopy) and histopathological studies. **Results:** Group III and IV animals showed a significant increase in serum biomarkers (AST, ALT, BUN, Creatinine) and apoptotic index. There was statistically significant decrease in viability count in treatment groups in comparison to the control group. Histopathology also revealed a significant hepatic damage and a moderate degree of changes in the renal tissue. **Conclusion:** Based on the above observations it was concluded that the administration of *Centella asiatica* @1,000 mg/kg b.wt for a period of 30 days may cause a significant damage to liver tissue in rats. **Significance & Impact of the study:** This study signifies the organ specific toxicity of *Centella asiatica*.

Key words: Mandookaparni, Toxicity, Apoptosis, Herbal Medicine

INTRODUCTION

India is very rich in natural resources and the knowledge of traditional medicine and the use of plants as a source of medicine is innate and very important component of the health care system. The Indian system of medicine has identified 1,500 medicinal plants of which 500 are commonly used. According to a recent estimate of WHO, 70-80% of the world population especially in developing countries relies on traditional medicine mostly plant drugs for their primary health care needs.[1]

India is the fourth largest producer of pharmaceuticals in the world and an important consumer of medicines too.

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Each day, new drugs are introduced and prescribed. The safety of drugs is of paramount importance to patients and health care professionals. The pharmaceutical industry has been an ethical and legal responsibility to ensure that the products they sell will not harm the patients they are intended for.

Patients taking prescription drugs and therapeutic herbs may be at risk for adverse drug-herb interaction, including that alters bio-availability and efficacy of prescription drugs. Drug interaction and adverse effects from herbal medicine are more likely to occur among patients who have chronic medical conditions, such as liver, heart, or kidney disease. Older patients have more comoribid illness and may be more susceptible to complications caused by herbal medicine. Several recent publications report on renal failure caused by Chinese herbs. The use of most herbal medicine is not evidence based and the risk clearly outweighs the benefit.

Centella asiatica (Sanskrit: - Mandooka parni, English: - Indian penny wort) is a medicinal plant with long history of therapeutic use in Indian system of medicine. It is a

creeping plant which has its origin in tropical and sub tropical climates. In south Asian countries such as India and Indonesia it has long history of therapeutic use, healing wounds and slowing the progression of leprosy. Furthermore, it is considered to prolong life and increase energy.

No information is available in regard to toxicological consequences of *Centella asiatica* with particular reference to their effects on apoptosis and marker enzyme levels in rats. In view of the dramatic and tremendous increase in the use of herbal drugs and in particular ever increasing exposure of humans to the *Centella asiatica* has become a reality. Despite these facts, the toxicological effects of *Centella asiatica* have not been investigated.

Therefore, the present study is designed in such a way to assess the toxic effects of *Centella asiatica* after short term oral administration in rats. The outcome of the study will provide necessary and useful information regarding the usage of *Centella asiatica* and there by improving its acceptability and safety in the world of alternative medicine.

MATERIALS AND METHODS

Collection of Plant

Centella asiatica was procured from the herbal garden maintained by the Department of Pharmacology and Toxicology, R.V.C, Ranchi, authenticated with botanist. Shade dried, powdered, muslinised, aerial parts were used along with gum acacia (binding agent) for oral administration in rats.

Experimental design

A total of 24 Wistar albino rats of either sex were randomly grouped in to 4 groups of 6 in each group. All the animals were allowed for acclimatization period of seven days before study. A set of two rats were housed in a propylene cage with 12 h: 12 h dark-light cycle, with feed and water ad libitum. The experimental protocol was approved by The Institutional Animal Ethical Committee (IAEC).

Rats in group I were fed with normal diet and kept as controls. Groups II, III and IV were fed with Centella asiatica @ the doses of 250, 500, and 1,000 mg/kg. b.wt. respectively for 30 days. Average body weight gain was measured at 10 days interval. All the animals were examined for clinical signs, gross behavioural changes, morbidity, and mortality once daily throughout the experimental period.

Sample collection

Blood samples were collected by heart puncture in to EDTA as well as plain vials at the end of the study. Various organs like spleen, heart, brain, liver, and kidney were collected and weighed and further samples were stored for further histopathological analysis.

Reagents and Chemicals

All the reagents and chemicals used in the experiment were procured from Sigma Chemicals Co.St. Louis, USA, E.Merck (India), SISCO Laboratories and, Erba Transasia bio-chemicals Ltd (assay kits for enzyme estimation).

Hematology

Total leucocytes count, RBC, Platelets, Hemoglobin, MCV & MCHC were analyzed on "Sysmex® KX-21 automated hematology analyzer".

Biochemical Estimation

Aspertate transaminase (AST), Alanine transaminase (ALT), Blood Urea Nitrogen (BUN), Creatinine level in the blood was measured (by kits from ERBA chemicals) on auto analyzer

Apoptosis Related Parameters

Ex Vivo Viability Count: Pieces of liver, kidney and heart were collected from the rats of different groups and tissues were further chopped and washed in chilled normal saline (0.9% NaCl solution). Then cells were disaggregated by gently rubbing and pressing the chopped tissue through a wire mesh (200-300gauge).[2] The cell suspension was adjusted to contain 1,00,000-1,20,000 cells per ml and were adjusted to viability count by following two methods.

Trypan Blue Dye Exclusion Test (TBDET)

One drop each of hepatocyte, kidney and heart cells suspension (containing approximately 10000-12000cells) were separately mixed with three drops each of trypan blue solution (0.2%). The unstained viable cells were distinguished from the blue stained dead cells particularly due to damage of cell membrane and increased cellular permeability. The unstained viable cells were counted under a microscope and the percentage calculated accordingly.[3]

Fluorescence Microscopy

25 µl of cell suspension was taken in a PCR tube and 5 µl of dye mixture (equal volumes of acridine orange (100 µgm/ml) + ethidium bromide (100 µgm/ml) were added. 10 µl of stained cell suspension was taken on a clean microscope glass slide and covered with a 22 mm sq cover slip. The cells were examined under a fluorescent microscope with a 40X to 100X objective and 200 total cells/slides were counted and normal versus apoptic cells were recorded. Apoptotic cells were identified based upon chromatin condensation, nuclear fragmentation, membrane

blebbing, cell shrinkage and apoptotic body formation and apoptotic index was calculated as

$$\text{Apoptotic index} = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells counted}} \times 100$$

DNA Fragmentation Assay

Briefly, the isolated leucocytes were washed with Tris-buffered saline (TBS) buffer (pH 7.6) and pelleted by centrifugation at 8000 rpm for 5 minutes. The cell pellet was treated with 200 µl of lysis buffer and vortexed. Supernatant was collected in another micro centrifuge tube (MCT). The pellet was again treated with 100 µl of lysis buffer and supernatant was collected similarly. 35 µl of 10% Sodium dodecyl sulfate (SDS) was added to the supernatant and kept at 37°C for 2 hrs. Digestion was done with 4 µl of proteinase-K (20 mg/ml) for 3 hrs at 50°C. The lysate was then extracted with equal volumes of phenol/CHCl3/isoamyl alcohol (25:24:01). DNA was precipitated with 0.5 volume of 10M Ammonium acetate and 2.5 volumes of absolute alcohol and incubated at 50oC for overnight. DNA was pelleted by centrifugation at 1200 rpm for 15 minutes. DNA was washed with 70% ethanol at 1200 rpm for 5 min. The pellet was dissolved in 20 µ lit of TE buffer.1 µl of RNase-A (10 mg/ml) was added and kept at room temperature for 1 hr. A 1.6% agarose gel (with ethidium bromide) was prepared with tri-borate- EDTA buffer (TBE) pH 8.0 and the sample was run at 60V for 1 hr. The presence of a ladder pattern indicates an apoptotic cell population.[4]

Necropsy and Histopathology

The postmortem examination of sacrificed rats was carried out for the presence of gross lesions, if any. Pieces of liver, kidney, heart, brain, lungs, and spleen were collected and fixed in 10% buffered formalin for histopathological examination. All the formalin fixed tissues were mentioned organs were routinely processed, cut at 5 mµ and stained with H&E stain.[5]

Statistical Analysis

Data was expressed as Mean ± Standard Error (SE) and examined for statistical significance of difference with student's 't' test, *p* value of <0.05 being considered statistically significant.[6]

RESULTS

Oral administration of *Centella asiatica* in Wistar albino rats in different doses (250, 500 and 1,000 mg/kg/b.wt) over a period of 30 days did not produce any clinical signs of toxicity, morbidity, mortality with no effect on gross behavioural effects. All the treated rats exhibited normal activities as that of the control group. All the four groups (control & treatment) of rats showed a steady gain in their body weight during the entire study period. (Table: 1). the changes in hematological parameters between the control and treatment groups were statistically significant but, all are in normal physiological range. (Table: 2).

Serum Bio-Markers: The effect of short-term exposure of 30 days oral administration of *Centella asiatica* resulted in a significant increase in the levels of ALT, AST in a dose dependent manner. The concentration of tissue total protein in groups II, III and IV were also significantly higher than the control group. The concentration of BUN and Creatinine in II, III and IV were also higher than the control group (Table: 3).

Table 1: Effect of *Centella asiatica* on the Body weight (gms) during the 30-Days oral administration in rats

Treatment	Dose (mg/kgwt)	O-Day	10-Days	20-Days	30-Days
Group-I	Control	104.32 ± 8.58	116.64 ± 12.91	120.37 ± 13.37	128.21 ± 13.68
Group-II	250	111.07 ± 7.78	123.47 ± 7.57	136.20 ± 8.38	140.78 ± 8.52
Group-III	500	125.05 ± 11.61	137.35 ± 6.96	140.93 ± 9.07	147.21 ± 6.21
Group-IV	1,000	119.53 ± 8.97	133.01 ± 9.70	139.48 ± 1.24	146.91 ± 1.01

Values are given as mean ± S.E *n* = 6 in each group * *P* < 0.05 as compared to control

Table 2: Effect of Centella asiatica on the Heamotological parameters after 30Days oral administration in rats

Parameters	Group-I	Group-II	Group-III	Group-IV
RBC (10^6/µl)	7.60 ± 0.31^a	7.36 ± 0.59^a	7.43 ± 0.32^a	10.03 ± 0.28^b
WBC (10^3/µl)	7.87 ± 0.30^a	6.08 ± 0.29^b	7.42 ± 0.60^a	6.60 ± 0.11^b
Hb (g/dl)	14.50 ± 0.69^a	13.27 ± 1.29^a	13.28 ± 0.48^a	18.35 ± 0.39^b
MCV (fl)	58.47 ± 0.83^a	53.58 ± 1.84^b	57.08 ± 0.76^a	56.62 ± 0.54^b
MCHC (g/dl)	30.85 ± 0.12^a	31.42 ± 0.19^b	32.00 ± 0.21^a	31.73 ± 0.37^a
PLT (10^3/µl)	565.67 ± 8.82^a	496.67 ± 25.02^b	551.83 ± 9.16^{ac}	634.00 ± 1.46^d

Values are given as mean ± S.E *n* = 6 in each group * *P* < 0.05 as compared to control

Table 3: Effect of *Centella asiatica* on the Marker Enzyme level in serum after 30Days oral administration in rats

Enzyme	Group-I	Group-II	Group-III	Group-IV
ALT (U/L)	56.62 ± 0.70[a]	159.80 ± 2.08[b]	179.95 ± 2.[c]	268.98 ± 12.24[d]
AST (U/L)	18.78 ± 0.5[a]	24.63 ± 0.26[b]	28.57 ± 0.54[c]	42.83 ± 1.8[d]
BUN (mg/dl)	19.69 ± 1.6[a]	23.45 ± 1.04[b]	26.91 ± 1.01[c]	31.13 ± 1.98[d]
CREA (mg/dl)	0.81 ± 0.04[a]	1.10 ± 0.06[b]	1.03 ± 0.11[ab]	1.54 ± 0.11[c]

Values are given as mean ± S.E $n = 6$ in each group * $P < 0.05$ as compared to control

Table 4: Effect of *Centella asiatica* on the TBDET of different vital organs after 30-Days oral administration in rats

Organ	Group-I	Group-II	Group-III	Group-IV
Liver	92.75 ± 0.85[a]	93.75 ± 0.63[a]	89.25 ± 0.85[a]	83.00 ± 0.91[a]
Kidney	94.50 ± 0.65[a]	94.25 ± 0.48[a]	91.50 ± 0.65[a]	90.25 ± 0.48[a]
Heart	94.00 ± 0.82[a]	95.00 ± 0.41[a]	94.00 ± 0.41[a]	93.50 ± 1.19[a]

Values are given as mean ± S.E $n = 6$ in each group * $P < 0.05$ as compared to control

Apoptosis related parameters were studied immediately at the end of the experiment and they are as follows

i. TBDET: The mean values of viable cell count in liver, kidney and heart tissues (%) by trypan blue dye exclusion test at the end of the trial period have been presented in Table-4. Viable hepatocyte count in group-III, IV was 89.25 ± 0.85 and 83.00 ± 0.91 (%) respectively. Which are significantly lower when compared with the group-I 92.75 ± 0.85 (%). Moreover, the viable count of kidney cells in group-I, II, III and IV was 94.50 ± 0.65, 94.25 ± 0.48, 91.50 ± 0.65 and 90.25 ± 0.48 (%) respectively.

Whereas, the viable count of cardiac cells in group-I, II, III and IV was 94.00 ± 0.82, 95.00 ± 0.41, 94.00 ± 0.41and 93.50 ± 1.19 respectively. No significance difference was recorded in the viable cell count of cardiac cells.

ii. Flourescence microscopy: The morphology of cells under fluorescence was characterized in Figures: 1 and 2. The apoptotic index was calculated and presented in the Table: 5. Apoptotic index of hepatic tissue was significantly increased in group-III and group-IV as compared to control group. However, the increase in apoptotic index was marginal in group-II which was fed with *Centella asiatica* @ 250 mg/kg b.wt.

Furthermore, kidney cells also recorded the increase in apoptotic index in all three treatment groups as compared to that of control group. There is no significant change/ increase in the apoptotic index was observed in the cardiac tissue.

iii. DNA fragmentation assay: DNA fragmentation assay of the leucocytes at the end of the experimental period showed clear intact pattern of DNA in all the four groups of rats (Figure: 3).

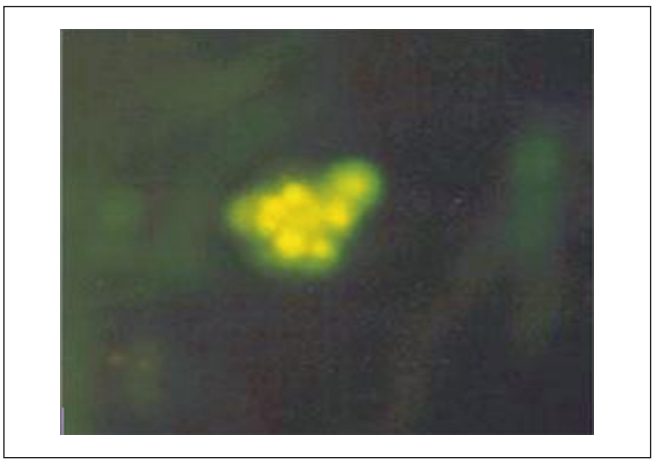

Figure 1: Microphotographs of hepatocytes showing cell shrinkage

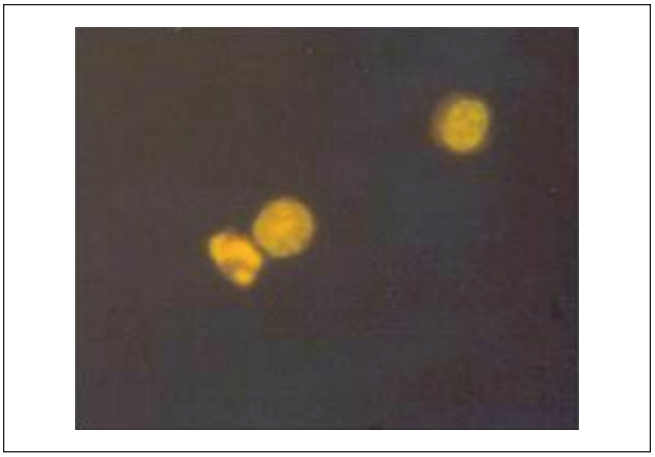

Figure 2: Microphotographs of hepatocytes showing Karyorhexis

Gross Pathology, Organ weight to body weight ratios and Histopathology

Effect of *Centella asiatica* on weights of liver, kidney, heart, spleen and brain (gms) of the rats are presented in the Table: 6. At necropsy, treatment groups did not show any gross

Table 5: Effect of Centella asiatica on the apoptotic index of different vital organs during the 30-Days oral administration in rats

Organ	Group-I	Group-II	Group-III	Group-IV
Liver	3.30 ± 0.33[a]	4.25 ± 0.38[a]	7.05 ± 0.50[b]	9.44 ± 0.96[c]
Kidney	1.38 ± 0.24	1.25 ± 0.14	2.13 ± 0.38	1.38 ± 0.24
Heart	1.75 ± 0.32	1.88 ± 0.31	1.63 ± 0.13	1.50 ± 0.20

Values are given as mean ± S.E $n = 6$ in each group * $P < 0.05$ as compared to control

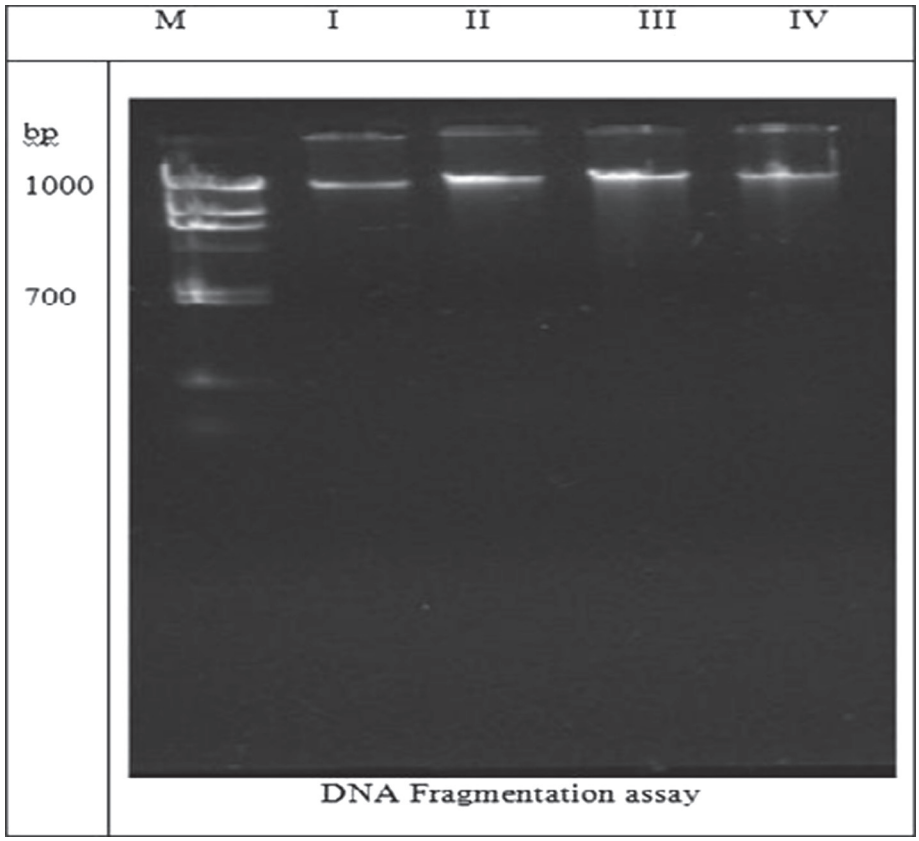

Figure 3: DNA fragmentation assay of leucocytes from the control and all the three treatment groups

Table 6: Effect of Centella asiatica on the Organ to body weight ratio (%) after 30- Days oral administration in rats

Organ	Group-I	Group-II	Group-III	Group-IV
Liver	4.83 ± 0.69	4.99 ± 0.37	5.02 ± 0.17	5.833 ± 0.47
Kidney	0.705 ± 0.15	0.733 ± 0.10	0.985 ± 0.22	0.968 ± 0.26
Heart	0.36 ± 0.04	0.32 ± 0.05	0.45 ± 0.05	0.47 ± 0.02
Spleen	0.38 ± 0.03[a]	0.44 ± 0.04[ab]	0.53 ± 0.03[b]	0.52 ± 0.03[b]
Brain	0.89 ± 0.06	0.78 ± 0.10	0.92 ± 0.07	0.89 ± 0.31

Values are given as mean ± S.E $n = 6$ in each group * $P < 0.05$ as compared to control

pathological lesions in any of the organs and no effect on organ weights and their ratios, except the spleen which has shown an increase in gross weight in a dose related manner.

Liver revealed consistent tissue alterations showing granular to vascular changes prominently in perilobular hepatocytes and comparatively milder in centrilobular hepatocytes. Some foci of the perilobular hepatocytes also showed perinuclear cytolysis as well as pycknotic nucei. Moreover there was marked infiltration of mononuclear cells and proliferation of bile ducts in portal areas. proliferation of central vein in portal areas and sinusoids in some focal areas were also consistently observed. (Figures: 4, 5).

Histopathological examination of kidney of the rats fed with *Centella asiatica* at a dose of 1,000mg/kg b.wt revealed

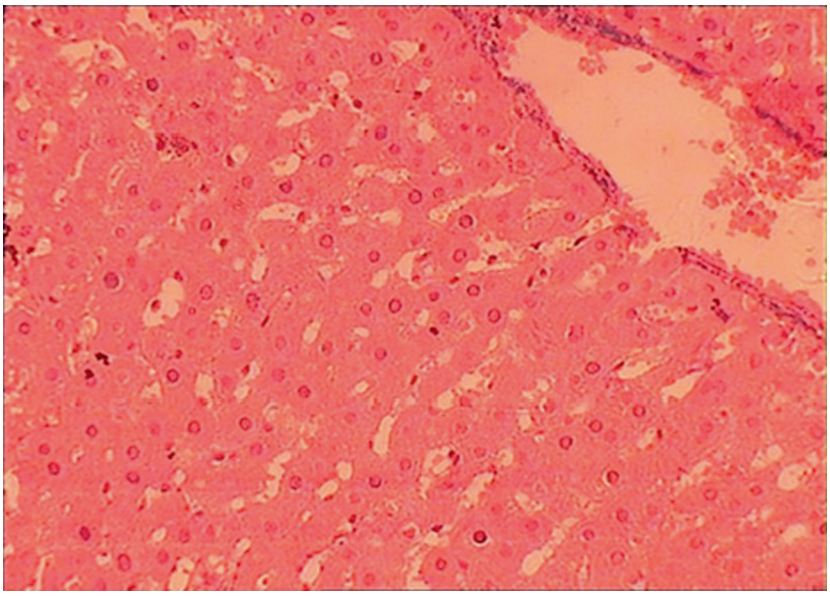

Figure 4: Micro photograph showing dilatation of sinusoids with presence of erythrocytes in them as well as more plump and active von kuffer cells

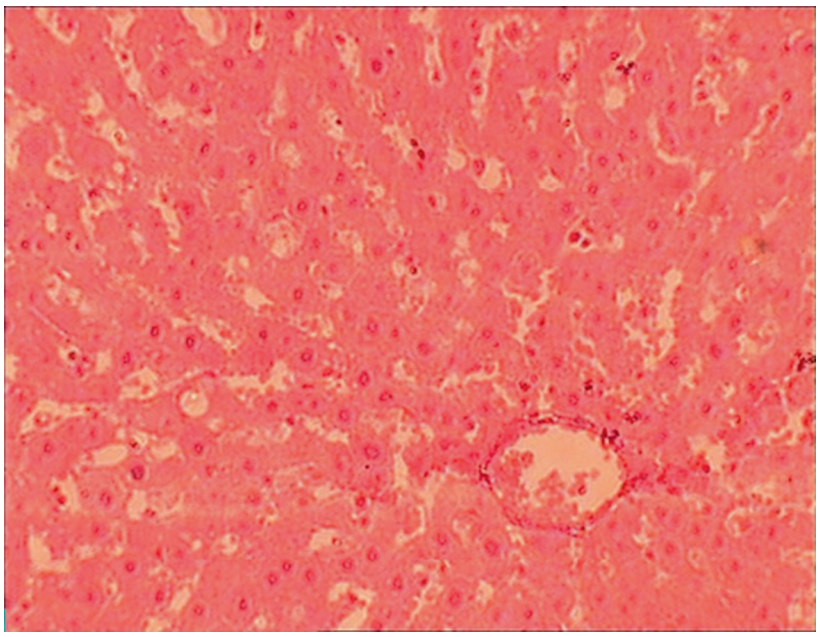

Figure 5: Micro photograph of hepatocytes showing granular vacuolar degeneration along with pycknotic karyorrhectic & nuclei

consistent tissue alteration showing granular, vacuolar and as well as desquamative changes in tubular epithelial cells of proximal convoluted tubules (Figure: 6).

At places, the lumen of the tubules showed albuminous precipitates. Mild congestion of intestinal blood vessels was also seen at some places. The bowman's area also showed congestive changes as well as hypercellularity.

The microscopic section of spleen showed hypercellularity in both red and white pulp, in rats fed with *Centella asiatica* at a dose rate of 1,000 mg/kg b.wt in comparison to the spleen of normal control group rats. (Figure: 7).Likewise, the spleen of rats fed with *Centella asiatica* at a dose rate of 500 mg/kg b.wt also showed mild increase in cellularity in red and white pulp.

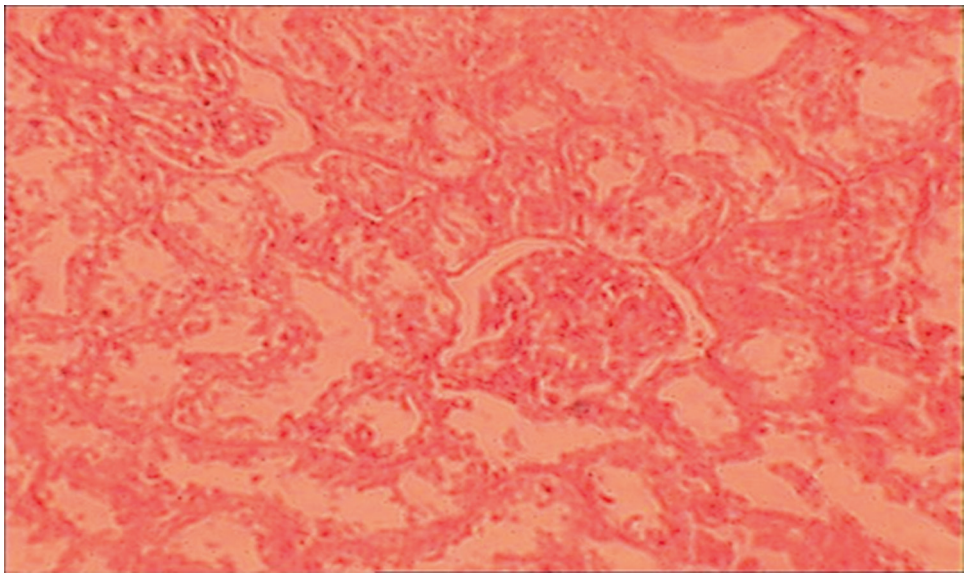

Figure 6: Micro photograph of rental tissue showing degeneration of tubular epithelial cells leading to desquamation at some places

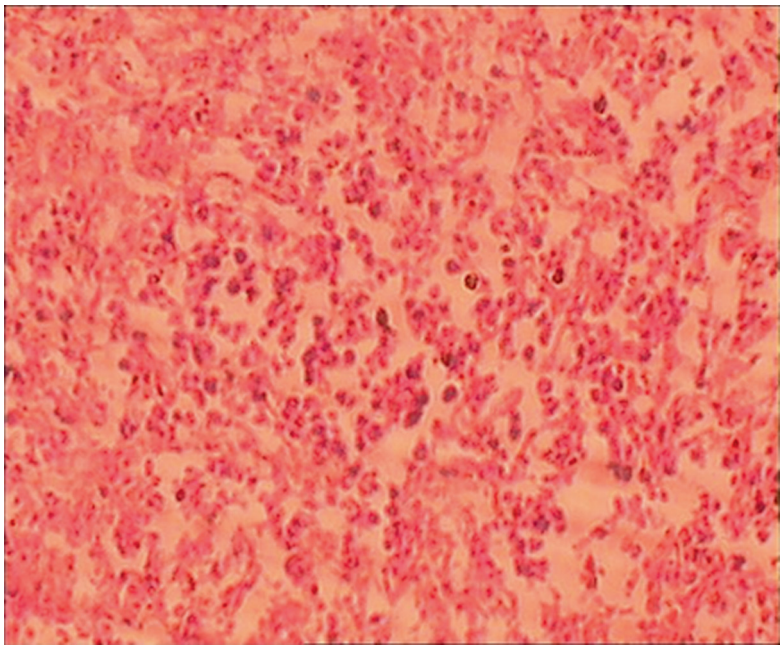

Figure 7: Spleen-Micro photograph of malphigian corpuscles showing increased mononuclear population

The microscopic section of brain and Heart resembled almost normal in all the groups of experimental rats which felt to reveal pathological changes of significance.

DISCUSSION

Centella asiatica administration at any of the tested dose levels did not affected the body weight gain. There were no abnormalities/ no adverse effects on hematology. The gross behavioral changes such as ataxia, catalepsy, pilo-errection, salivation, hyperactivity, cyanosis, lacrimation, righting and pinnal reflexes were found to be normal throughout the entire experimental period.

Liver being the main detoxification organ and kidney the major excretory organ, these are very susceptible to the toxicities by drugs. Damage to liver is known to result in cellular changes in the tissues and alterations in activities of enzymes in tissues and serum.[7] Damage of the liver results in entry of these particular enzymes in to the circulation. Therefore, the increased serum levels of GOT

and GPT are used as indicators of liver damageobserved significant elevation in the serum levels of aspertate and alanine transaminase after oral administration of *Centella asiatica* in human patients.[8]

The extent of liver injury is associated with increased serum levels of AST, ALT. The injuries may be acute/ chronic, reversible or irreversible. Of these enzymes, ALT is thought to be more specific to hepatic injury because it is mainly present in the cytosol of the liver cells and is in low concentrations elsewhere.

Renal parameters were also evaluated in the present study. Kidney eliminates waste product of metabolism from the body. In renal failure, waste products particularly nitrogenous substances like Non protein nitrogen, urea, uric acid accumulates. Creatinine is the least variable nitrogenous constituent of blood. Creatinine increases in early nephritis & in chronic hemmorrhgic nephritis with uremia. In the present study, *Centella asiatica* increased both blood urea nitrogen and Creatinine values in a dose dependent manner.

Any significant loss of a cell population or particular cell type may result into dysfunctioning of an organ. Induction of apoptosis could impair the steady' state kinetics of healthy tissues in an organism resulting in deregulatory cellular responses.[9, 10] Apoptosis can be assessed by biochemical (DNA fragmentation assay) and morphological (fluorescence microscopy) assays.

TBDET viability count method was designed for ascertaining the initial event of cellular injury is based on the fact that living cell membrane is able to selectively exclude certain substances where as dead cells permit these substances to enter in side. The trypan blue is a dye which is also excluded by the living cells whose membrane does not allow the dye to enter inside the cell. However, cells whose membranes become permeable, trypan blue enters in side the cell and stains them. In this way, this dye distinguishes between permeable and non permeable cells there by identifying the live and dead cells.[11] Liver tissue of group-III and IV rats showed a significant decrease in the viability count when compared to normal control group.

Flourescence Microscopy – using acridine orange (AO) and Ethidium bromide visualized the characteristic features of apoptosis i.e. cell shrinkage and karyorhexis in the hepatocytes.

Histopathology

The consistent development of prominent perinuclear cytolysis, pycknotic nuclei in perilobular hepatocytes, marked infiltration of mononuclear cells and proliferation of bile ducts in portal areas, proliferation of central vein in portal areas and sinusoids in some focal areas were indicating the hepatotoxicity of the *Centella asiatica*.

Degeneration or necrosis of epithelial cells of the renal tubules, albuminous precipitates in the lumen, indicate nephrotoxicity of *Centella asiatica*.The hypercellularity and Hyperplasia of spleen could be correlated to immunosuppressive effect of *Centella asiatica*. No significant microscopic alterations could be seen in brain, heart and lungs.

CONCLUSION

Centella asiatica in the doses (250,500 and 1,000 mg/kg bwt) used in the present study over a period of 30 days produced no apparent toxicity and alterations in the body weight gain. But significant increase in the weight of the spleen in the group of rats fed with *Centella asiatica* @ 1,000 mg/kg b.wt was observed. The level of ALT, AST in the serum which indicates the liver functionality was significantly raised in all the three treatment groups. And the increase in BUN and CREATINNIE levels in the serum suggested the possible damage of renal tissue. Though the hematological parameters are showing significant change between groups, all those values are in normal physiological range. *Centella asiatica* @ 1,000 mg/kg b.wt caused induction of higher levels of apoptosis in hepatic tissue and a marginal increase in the renal tissue.

The result of present study clearly indicated that when *Centella asiatica* was orally administered in different doses in rats for 30 days the various biochemical parameters of the rats were affected. The apoptosis induced by *Centella asiatica* in liver shows a promising way for future studies on its role in tumor management. More detailed studies with multiple parameters on dose time relationship in various other species of animals will help in completion of the *Centella asiatica* toxicity on biological systems.

Acknowledgements: We are thankful to Dr. Anita Saxeena, College of BIOTECHNOLOGY, BAU, Ranchi for permitting us to avail their lab facilities.

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Phytochemical Screening and Antioxidant Activity of essential oil of Eucalyptus leaf

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ABSTRACT

Barks, roots, fruits, buds, leaves and other parts of plant are considered as source of essential aromatic oils to cure several ailments. The antioxidant property has been shown to be important in recovery from several diseases. The essential oil extracted from eucalyptus leaves was tested for phytochemical analysis and antioxidant activities. The Eucalyptus oil extracted from the leaves of *Eucalyptus globulus* family Myrtaceae was screened for the presence of phytochemicals and their effect on 2, 2-Diphenyl-1-picryl-hydrazyl radical (DPPH) and Nitric oxide free radical. Phytochemical screening of the plants showed the presence of flavonoids, terpenoids, saponins and reducing sugars. *Eucalyptus globulus* is not having any cardiac glycosides and anthraquinones. The free radical scavenging activity of the different concentrations of the leaf oil (10, 20, 40, 60 and 80% (v/v) in DMSO) of *E. globulus* increased in a concentration dependent fashion. In DPPH method, the oil in 80% (v/v) concentration exhibited 79.55 ± 0.82%. In nitric oxide radical scavenging assay method, it was found that 80% (v/v) concentration exhibited 81.54 ± 0.94% inhibition. It was concluded that leaf oil is potent inhibition of free radicals.

Key words: Eucalyptus globules, Antioxidants, DPPH, NO, Free radical.

INTRODUCTION

Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules.[1] Once formed these highly reactive radicals can start a chain reaction. The chief danger of free radical is that when they react with important cellular components such as DNA, or the cell membrane, the cell functions poorly or die.[2] To prevent free radical damage the body has a defense system of antioxidants.[3-4] Antioxidants are intimately involved in the prevention of cellular damage thus inhibits the common pathway for cancer, aging and a variety of diseases.[5-6] In order to prevent this cellular damage, apart from body natural defense system, some herbal extracts/oils/formulations has been proved as important antioxidant agents.[7-9]

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Several researchers have shown that aromatic and medicinal plants are sources of diverse nutrient and non nutrient molecules, many of which can cure several ailments as pain, pyrexia, hypertension, kidney stone and sunburn diseases.[10-12] In traditional books of Ayurveda, the eucalyptus oil has been strongly recommended for antimalarial action, antiseptic action, antispasmodic, antiviral, astringent, balsamic, decongestant, deodorant, depurative, diuretic, expectorant, febrifuge, hypoglycaemic and rubefacient stimulant action.[13-14] The literature survey revealed that eucalyptus oil antioxidant activity has not been proved scientifically, so the phytochemical investigation and antioxidant properties were subjected for evaluation. It is also used as a stimulant and antiseptic gargle and if locally applied, it impairs sensibility.[15-16]

E. globulub is a large tree attaining a height of 300 ft. or more, with a clean straight bole under forest conditions, but often tending to branch freely when grown in the open. Leaves on juvenile shoots are opposite, sessile, cordate-ovate and covered with a bluish white bloom.[17] The adult leaves are alternate, lanceolate, 6-12 inch long and 1-2 inch broad.[18] Stems of the seedlings and coppice shoots are quadrangular. Flowers are in cymose panicles.[19] The

Figure 1: Leaves of *E. globulus*

medicinal properties of Eucalyptus reside in its oil, which is extracted from the fresh leaves.[20] Eucalyptus leaf is an approved remedy of the German Government's Federal Institute for Drugs and Medical Devices (Commission E) for catarrhs of the respiratory tract. Eucalyptus oil is generally compared to menthol because of its tendency to acts on receptors in the nasal mucosa, which help to alleviate nasal congestion.[21]

MATERIALS AND METHOD

Plant material and extraction

Eucalyptus globulus fresh leaves were collected in the month of February 2010 from garden of IFTM campus, Moradabad. The leaves were identified, authenticated and voucher specimen (No. IFTM/EL/1/2010) is kept in herbarium section of Deptt. of Pharmacognosy, IFTM, Moradabad, India for future reference. The collected leaves were properly washed with tap water, cutted into small pieces (total weight 100 gm) and packed in distillation flask of Clavenger's apparatus with sufficient quantity of water and few pieces of porcelain chips to avoid bumping during distillation. The extraction was continued for six hours. The eucalyptus oil was collected from graduated receiver and purified by anhydrous sodium sulphate for removing water traces.

The oil obtained was diluted with DMSO at the concentrations of 10, 20,. 40, 60 and 80% (v/v) and were used for *in vitro* antioxidant studies following DPPH and Nitric oxide free radical scavenging assay.

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH), Curcumin,EDTA, Ascorbic acid, perchloric acid were purchased from Sigma, St. Louis, USA. All other chemicals and reagents as sulphanilamide, naphthylethylene diamine dihydrochloride, sodium nitroprusside, methanol and phosphoric acid used were of analytical grade.

Phytochemical Screening of Eucalyptus oil

The oil extracted was screened for the presence of carbohydrate, tannins, phenolic compounds, alkaloids, anthraquinones, cyanogenetic glycosides, saponin glycosides and steroidal nucleus by using the reported methods.[22]

In-vitro antioxidant activity

DPPH free radical scavenging activity[23-24]

Ascorbic acid (10 mg) was dissolved in methanol and diluted in a way to get the final concentration of 100 µg/ml and it served as positive control. Various dilutions of Eucalyptus oil were prepared by dissolving it into DMSO and finally concentration of 10, 20, 40, 60 and 80% (v/v) were prepared. One ml of 100 µM DPPH in methanol was mixed with equal volume of the diluted eucalyptus oil solution in phosphate buffer (pH 7.4), mixed well and kept away from light for 20 min. The prepared test solutions mixture was protected from light by wrapping the test tubes with aluminum foil. The absorbance at 517 nm (DPPH) was monitored in presence of different concentrations of extract. Absorbance of blank solution was also measured to determine the absorbance of DPPH prior to start of reaction with the eucalyptus oil. Percentage inhibition of the radicals was calculated according to formula as per equation 1.

$$\%\text{Inhibition} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Nitric oxide radical scavenging activity[25-26]

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was it was measured by the Griess reagent. SNP in saline phosphate buffer generates NO that can be estimated by the use of Griess Reagent. SNP (10 mM) in phosphate buffer saline (PBS) was mixed with different concentration of oil 10, 20, 40, 60 and 80% (v/v) and the resulting mixture was subjected for incubation at 25°C for 180 minutes. The samples of the above mixture were reacted with Griess reagent which is comprised of 1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid. The absorbance of the colored component formed during the diazotization reaction of nitrite ion with sulphanilamide and then coupling with naphthylethylenediamine dichloride was measured at 546 nm and also the absorbance of ascorbic acid in similar process after reacting with Griess reagent was measured. The ascorbic acid absorbance served positive control.

$$\text{Nitric Oxide scavenged (\%)} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control}$ = Absorbance of control reaction and A_{test} = Absorbance in the presence of the samples of eucalyptus oil. Each experiment was carried out in triplicate and results are expressed as mean % scavenged activity ± SD.

Table 1: Phytochemical screening of oil obtained from leaves of *Eucalyptus globules*

Phytochemical Test	Result
Alkaloids	–
Flavonoids	+
Saponins	+
Steriods	–
Glycosids	–
Terpenoid	+
Tannins	–
Reducing sugar	+

RESULTS AND DISCUSSION

Phytochemical evaluation of Eucalyptus oil showed the presence of flavonoids, terpenoids, saponins and reducing sugars. (Table 1).

Eucalyptus oil was found to be potent free radical scavenger. The percent inhibition was found in concentration dependent manner almost same in both assay methods. The IC_{50} value of eucalyptus oil was found to be 33.72 and 29.23 (v/v) with DPPH and NO respectively (Table 3). The presence of flavanoid constituent upon phytochemical screening enhanced the chances of antioxidant activity and same was proved by these assay methods.[27]

The *in vitro* antioxidant activity of Eucalyptus oil was performed by DPPH and nitric oxide free radical scavenging method. For this, five concentrations –10, 20, 40, 60 and 80% v/v (20 µl of each sample) of Eucalyptus oil were used. The scavenging effects of different concentrations of oil and Ascorbic acid (100 µg/ml) in the DPPH and NO method are illustrated in Table 2.

The eucalyptus oil with 80% (v/v) concentration exhibited 79.55 ± 0.82% activity whereas standard exhibited 88.11 ± 0.71 inhibition by DPPH method. The scavenging effects of all other concentration were found to be relatively less than ascorbic acid. The concentration 80% (v/v) by NO method also showed all most similar percent inhibition. The concentrations 60 and 80% (v/v) shown statistically significant free radical scavenging activity ($p < 0.05$) by DPPH and Nitric oxide methods. The IC_{50} was found at 33.72 and 29.23 (v/v) with DPPH and NO respectively for Ecalyptus oil. The standard used for both assay method was ascorbic acid (concentration 100 µg/ml) which shown 88.11 ± 0.71 and 91.24 ± 1.44 percent inhibition in two assay methods performed.

CONCLUSION

On the basis of the results obtained in the present study, it WAS concluded that a eucalyptus oil extracted from fresh leaves of *E. globules* showed presence of flavonoid, saponins, terpenes and reducing sugars. The positive test especially for flavonoid (Phenolic compound) in oil supported the idea that eucalyptus oil is having free radical scavenging potential. The in vitro assays by DPPH and NO assay

Table 2: % Inhibition of free radicals by different concentrations of eucalyptus oil

S. No.	Method	Concentration and its % inhibition					
		10 v/v	20 v/v	40 v/v	60 v/v	80 v/v	Standard#,§
1	DPPH assay	15.12 ± 0.46	33.95 ± 1.46	58.12 ± 0.71	66.70 ± 1.44*	79.55 ± 0.82*	88.11 ± 0.71
2	NO assay	12.60 ± 1.05	28.61 ± 0.56	61.52 ± 1.50	75.20 ± 0.21*	81.54 ± 0.94*	91.24 ± 1.44*

#in all cases, ascorbic acid was used as standard (positive control). *$p < 0.05$ when compared to control. §the ascorbic acid used to compare was in concentration 100 µg/ml

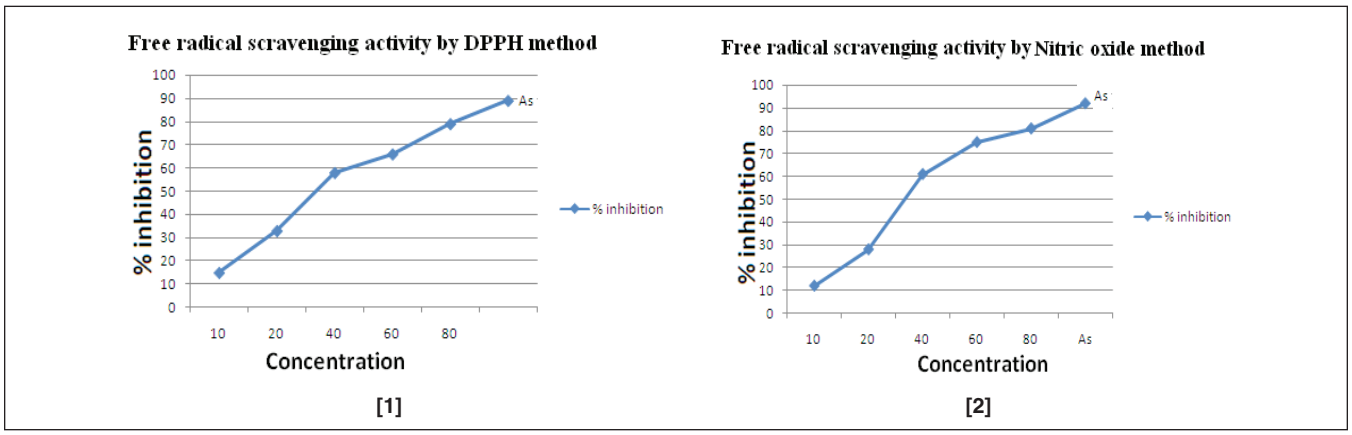

Figure 2: [1] Representation of free radical scravenging activity by DPPH method(n = 3), and -[2] represents free radical scravenging activity by NO assay method(n = 3). (As = Ascorbic acid).

methods indicated that the oil of this plant is a potent source of natural antioxidant, which may be helpful in preventing the progress of various oxidative damages on cellular functions and also helps to eradicate several free radical born diseases. The particular components which are responsible for the antioxidant activity are currently unknown and further focus is needed to identify the actual responsible chemical constituent and its structure.

ACKNOWLE DGMENTS

Authors are grateful to Dr. R. M. Dubey (Managing Director, IFTM) for continuous encouragement and providing necessary chemicals and facility in order to complete this research work.

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Antimicrobial activity of some bryophytes (liverworts and a hornwort) from Kolhapur district

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ABSTRACT

The *in vitro* antibacterial activity of the extracts of the bryophytes was assessed using agar-well diffusion method against three bacterial strains. The bryophyte extracts were prepared in methanol and dichloromethane. Inhibition of bacterial growth was compared with that of ampicillin and tetracycline as positive control and solvents as negative control. The methanol and dichloromethane extracts of *Plagiochasma intermedium, Asterella wallichiana* and *Targionia hypophylla* had potential activity against all the microorganisms tested. The dichloromethane extract of *P. intermedium,* methanol extracts of *A. wallichiana* and *T. hypophylla* also showed good results. Among all the bryophytes investigated for *in vitro* antimicrobial activity the extracts of *T. hypophylla* showed most promising results than that of all others while the extracts of *Cyathodium cavernarum* was rather inactive.

Key words: antibacterial, *Anthoceros, Asterella, Plagiochasma, Targionia*

INTRODUCTION

In contrast to the extensive utilization of substances from higher plant sources, bryophytes have rarely been considered as a source of substances useful for human beings because they are very small and difficult to collect in large amounts as pure samples. However, they have been used as medicinal plants to cure cut, external wounds, bacteriosis, neurasthenia, pulmonary tuberculosis, etc. Extracts of many bryophytes have been shown to possess varying levels of antibacterial and anticancer activities *in vitro*.[1-4] The classes, liverworts and hornworts of bryophytes represent an interesting groups. These are very small and ubiquitous plants. An interesting feature of bryophytes is that they are neither attacked by either bacteria or fungi nor damaged by insects or snails.[5] Considering such observations bryophytes might have in use as medicinal plants for more than 400 yrs. in China, Europe and North America to cure various types of diseases. The Chinese and the native Americans have used various moss species in the form of paste and applied as poultice. Chinese traditional medicine names 40 kinds of bryophytes that have been used to treat illness of cardiovascular system, tonsillitis, bronchitis, tympanitis, cystitis as well as skin diseases, cuts, burn and wounds. In India the burnt ash of mosses mixed with fat and honey is used as an external application for cuts, burns and wounds in the Himalayan regions. The liverworts *Marchantia polymorpha* is also used as medicine for boils and abscesses. The rosette forming *Riccia* spp. is used as an external application to cure ringworm. When bryophytes screened for antitumor activity it was found that number of mosses and liverworts were active.[6] Antimicrobial and antifungal activities have been reported for wide range of liverworts.[7-9] These activities are due to biologically active compounds present in them particularly lipophylic extracts of several liverworts namely *Bazzania, Frullania, Marchantia, Plagiochilla, Porella and Reitdulla spp.* show antibacterial and antifungal activities. According to some scientists non-ionized organic acids and polyphenolic compounds might contribute the antibiotic properties of bryophytes and it is found that mosses strongly inhibit one or both positive and negative bacteria.[10-11] Gupta and Singh[12] have reported a high occurrence of antibacterial activity in extract of *Barbula* spp. reaching as high as 36.6 % whereas it is only half that in *Timmiella spp* (18.8%). Asakawa and Heidelberger[13] isolated 3 prenyl bitibbenzyl from *Redula spp* and showed that it could inhibit growth of *S. aureus* at the concentration of 20.3 μgml^{-1}. A wide range of antibacterial activity has been observed a in nearly all bryophytes tested.[14-15] Antimicrobial activity has been reported in the extracts of many liverworts.[16]

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The existence of high antibacterial activity in case of liverworts may be due to the biologically active compounds in them. The methanol extract after chromatographic separation gave cyclic bis-bibenzyles, marchantin A and analogues, one of which showed antimicrobial activity against *B. cereus* (MIC 12.5 μgml^{-1}), *B. megaterium* (25 μgml^{-1}), *B. subtilis* (25 μgml^{-1}) and *S. aureus* (3.13-25 μgml^{-1}).[17] Some of the active ingredients that have been isolated and identified are Polygodial from *Porella*, Norpiguisone from *Conocephalum conicum* and Lanularin from *Lanularia cruciata*.[18] 4-hydroxy-3'methoxybibenzyl and α- and ß-pinine-alloromadendrin in *Plagiochila stevensoniana* proved to inhibit *Candida albicans*, and *B. subtilis*.[19] A literature search revealed very little studies on the antimicrobial activity of Indian bryophytes especially those from bryophytes from this region. Therefore the present investigation was undertaken.

MATERIAL AND METHODS

Plant Material

Five different bryophytes viz. *P. intermedium, A. wallichiana, T. hypophylla, C. cavernarum* and *A. subtilis* with the rhizoids were collected from the Panhala region of Kolhapur district, Maharashtra state in the months of August and September. The care was taken to harvest the pure and uniform patch of particular species of bryophyte to be tested.

Fresh gametophytic samples of said bryophytes were extensively washed with tap water and distilled water and surface dried on blotting paper. Any dirt and senescent parts of plants were removed during the washing process.

5 g air dried plant material along with rhizoids was powdered in mortar with pastle and soxhlet extracted in 150-160 ml of two different solvents i.e. dichloromethane and methanol at 40°C. Methanol extract was evaporated to 30 ml under vacuum pressure at 40°C. While dichloromethane extract was dried and redissolved in 30 ml of acetone.These extracts were used for further studies.

Test microorganisms

Three bacterial strains were procured from NCIM, NCL, Pune. *In vitro* antibacterial activity was tested against 2 Gram – positive bacteria *Staphylococcus aureus* (NCIM 5021), *Bacillus subtilis* (NCIM 2010) and a Gram-negative bacterium *Escherichia coli* (NCIM 2089).

Determination of antimicrobial Activity

Plant extracts were tested for antimicrobial activity through agar-plate diffusion method[20] using 100 µl of suspension of the test microorganisms. Bacterial strains were grown on nutrient agar plates. Sterile nutrient agar plates were prepared, 48 hrs cultured suspensions were made and inoculated on sterile agar medium in the respective culture plates. The 10 mm sterile cork borer was used for making wells. The extracts, 0.1 and 0.2 ml were added in assay well carefully. 50 μgml^{-1} tetracycline and ampicillin (Hi-Media) which served as +ve control and methanol and acetone (Hi-Media) used as –ve control were added in the wells. All the plates were kept at low temperature for 1-1½ hrs for sample diffusion and incubated at 35°C for 48 hrs. After incubation, the zone of inhibition was measured.

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Adult thalli of said bryophytes (liverworts and hornwort) collected from Panhala region underwent extraction with dichloromethane and methanol. Inhibition of bacterial growth was compared with those of tetracycline and ampicillin. The antibacterial activity results are shown in [Table 1]. Our findings indicated that all the extracts of bryophytes studied showed varying levels of *in vitro* antibacterial activity against all the test bacteria. Of the bryophytes investigated both extracts of three liverworts, *T. hypophylla, P. intermedium* and *A, wallichiana* showed promising results while that of *T. hypophylla* showed very good antibacterial activity. *C. cavernarum* and the hornwort, *A. subtilis* extracts had more or less the same levels of antibacterial effects, whereas, *C. cavernarum* extracts were rather inactive against all the microorganisms under investigation. It may be due to the very thin, papery structure of thallus of *C. cavernarum* which contains very low concentrations of active compounds against microorganisms.

The results revealed that methanol extract of bryophytes had *in vitro* greater potential for antibacterial activity than that of dichloromethane extract against *E. coli* and *B. subtilis*. The dichloromethane extract had greater antibacterial activity against *S. aureus*. It was observed that *B. subtilis* was most susceptible than rest of the bacteria tested with maximum inhibition zone of 14 and 11mm being against methanol extract of *T. hypophylla* and *A. wallichiana* respectively and 12 and 11mm against dichloromethane extracts of *P. intermedium* and *T. hypophylla* respectively. But *S. aureus*, a Gram-positive bacterium gave weak response.

In case of *E. coli* no response was observed to the *C. cavernarum* extracts and same was true with that of dichloromethane extract for *B. subtilis*. In case of both the *A. subtilis* extracts and the lower concentrations of *C. cavernarum* extracts same results were observed i.e. no antibacterial activity was observed against *S. aureus*. Our findings showed that extracts of liverwort had interesting activity against Gram-positive and Gram-negative bacteria. It is common that the sensitivity of

Table 1: Antibacterial activity of methanol and dichloromethane extracts of some bryophytes (expressed as inhibition zones in mm)

Plant /substance	Test organism	Inhibition zone (mm)			
		Concentration of extract (µl)			
		Methanol Extract		Dichloromethane Extract	
		100	200	100	200
P. intermedium					
	E. coli	6.0 ± 2.0*	9.67 ± 0.5*	4.67 ± 0.5	10.67 ± 0.5*
	B. substilis	4.67 ± 2.0	7.67 ± 2.08	8.67 ± 5.50	12.33 ± 4.04
	S. aureus	5.0 ± 1.0	7.67 ± 1.52*	5.0 ± 1.0	9.0 ± 1.0
A. wallichiana					
	E. coli	6.67 ± 1.53	12.33 ± 0.58*	0.0	0.67 ± 1.15
	B. substilis	7.0 ± 0.0	11.00 ± 1.73	0.0	3.67 ± 0.58
	S. aureus	5. 67 ± 1.15	7.67 ± 2.08	2.67 ± 1.52	4.67 ± 2.31
T. hypophylla					
	E. coli	8.0 ± 1.0*	12.3 ± 2.89*	4.67 ± 0.58	8.0 ± 1.73*
	B. substilis	9.0 ± 1.0*	14.33 ± 0.58*	6.33 ± 1.54	11.33 ± 2.5*
	S. aureus	3.67 ± 1.53	9.67 ± 0.58*	5.67 ± 2.08	12.0 ± 1.0*
*C. cavern*arum					
	E. coli	0.0	0.6 ± 0.57	0.0	0.0
	B. substilis	0.33 ± 0.57	1.67 ± 1.57	0.0	0.0
	S. aureus	0.0	0.33 ± 0.58	0.0	1.33 ± 2.31
A. subtilis					
	E. coli	0.0	3.33 ± 0.58	2.33 ± 4.04	6.67 ± 6.42
	B. substilis	0.67 ± 1.15	1.67 ± 1.55	0.33 ± 0.58	3.0 ± 1.73
	S. aureus	0.0	0.0	0.0	0.33 ± 0.58
Methanol					
	E. coli	0.0			
	B. substilis	0.0			
	S. aureus	0.0			
Acetone					
	E. coli	0.0			
	B. substilis	0.0			
	S. aureus	1.0 ± 0.0			
Tetracyclin					
	E. coli	22.33 ± 0.58			
	B. substilis	11.67 ± 1.25			
	S. aureus	15.0 ± 1.0			
Ampicillin					
	E. coli	17.77 ± 0.58			
	B. substilis	16.66 ± 0.58			
	S. aureus	1.00 ± 0			

Values are mean ± SD. $n = 3$ in each group. *$P < 0.001$ compared to negative control (Tukey test). The activity was assayed by disc diffusion method.

Gram-negative bacteria is generally higher than that of Gram-positive ones. This is important from medicinal standpoint because antibacterial substances which are normally employed in therapy are active chiefly against Gram-positive.

The present study reported that liverworts showed varying levels of activity against all the three test bacteria. This suggests that the extracts of three liverworts, *T. hypophylla, P. intermedium, and A. wallichiana* in both the solvents have a broad spectrum of activity although the degree of susceptibility could differ between different organisms. Thus the above extracts are worthy of further investigation for their use as antibacterial agents. There is a need of purification and characterization of active principle(s) which can help in new drug development.

ACKNOWLEDGEMENT

The first author is thankful to the Co-ordinator, Department of Agrochemicals and pest management, Shivaji University, Kolhapur for providing necessary laboratory facilities.

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Comparison of Fresh with Dry Extracts for Antibacterial Activity of *Vigna radiate* L. on Pathogenic Bacteria

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ABSTRACT

Introduction: *Vigna radiat*a L. is common vegetable plant cultivated all of the world. It's belonging to the family Leguminosae (Fabaceae**). Methods:** To investigate for antibacterial activity of *Vigna radiata*, fresh and dried extracts of plant parts were tested on five strains of bacteria using standard well agar diffusion method. **Results:** Dried extracts showed more effective action on tested bacteria than of fresh extracts. Extracts prepared from dried stem and root exhibited better antibacterial activities than those prepared from fresh plant. Furthermore, there was no difference in the activity of ethanolic and aqueous extracts on isolated bacteria. Both of gram positive and negative bacteria showed approximately the same ratio of susceptibility to each part of plant.**Conclusions:** *V. radiata* has a potential antibacterial activity on clinically isolated bacteria. Dried extracts showed more effective action on tested bacteria than fresh extracts.

Key words: aqueous extract, ethanolic extract, Vigna radiata**,** bacteria

INTRODUCTION

For testing antimicrobial activity of any suggesting plant, preparation of extract from fresh parts is preferred due to retain the components of plant in active state.[1] This is not always available because selected plants needed to collect from so far distance from the place of actually extraction work. Thus, plant must be dried until extraction time.

Many studies are designed to compare between antimicrobial activities of fresh and dry plant. Results are variable to determine which state of plant is effective against organisms. Pepeljnjak et al[2] found that extracts prepared from fresh leaves of *Pelargonium radula* have significant higher antimicrobial activity than those prepared from dried leaves. Fresh fruit shell of Pomegranate is also has antibacterial effect on the bacterium *Ralstonia solanacearum* than dry fruit shell.[3] Whereas, Goyal et al[4] demonstrated that dry powder extracts of all *Catharanthus roseus* parts showed more antibacterial activity than extracts prepared from fresh parts.

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Vigna radiata L. or also called mungbean is belonging to the family Leguminosae (Fabaceae). It is very important economic plant through its contents of valuable nutrients. Furthermore, *V. radiata* facilitates the nitrogen fixation in soil by producing nodules on its root in combines with *Rhizobium*.[5] However, *V. radiata* contains within its species many genotypes resistance to bacterial infection.[6]

To determine the variation between antibacterial activity of fresh and dry parts of *Vigna radiate*, extracts of these plant parts was tested on many bacteria. Furthermore, this study tried to detect the differences between the activity of fresh and dry plant against bacteria.

MATERIALS AND METHODS

Plant preparation

Seeds of *Vigna radiate* L. (Fabaceae) were obtained from institute of agriculture of Karbala province (Iraq). Cultivation was performed in prepared field with suitable soil during July to August 2009. Mature plants were harvested and washed under running tap water. Root nodulation and damaged parts were removed. Plant materials (leaves, stem, and root) were separated and washed once again with distilled water.

Plant extracts

Extraction was performed by two different modes: (1) Extraction of fresh plant materials without drying and (2) Extraction after each plant part was air-dried

under room temperature. The 20 g of grounded plant part was extracted in electrical blender with 100 ml of ethyl alcohol (70%) for obtaining ethanolic extract and with sterilize distilled water for obtaining aqueous extract for 5 min and left for 1 h. Extracts were filtered through sterilized gauze and concentrated to dryness at room temperature.

Test organisms

Tested pathogenic bacteria were clinically isolated from AL-Hussein general hospital in August 2009. Five strains of bacteria were isolated. Strains were diagnosed using API 20 system (Biomérieux, Netherlands-France). The isolated bacteria were: *E. coli, Staphylococcus aureus, Klebisella pneumoniae, Proteus vulgaris,* and *Bacillus subtilis,*.

Antibacterial assay

Standard culture of bacteria for antibacterial assay was prepared by culturing of isolated bacteria in Mueller-Hinton broth (HiMedia, Mumbai-India) to become equivalent to 0.5 MacFarland standard (reading to 1×10^8 cfu/ml) and diluted 1:10.

All extracts were sterilized by sterile membrane syringe filter (pore size 0.45 µm, manufactured by Pall Life Science). Well agar diffusion recommended by NCCLS[7] was used. A well of 6 mm was performed in plate with Mueller-Hinton agar (HiMedia, Mumbai-India) inoculated with isolated bacterial strains. Various concentrations (3.125, 6.25, 12.5, 25, 50 mg/ml) of fresh and dried extracts were prepared in sterilize distilled water. Each well filled with 50 µl of specific concentration of extract. Cefotaxime sodium (30 µg) supplied by KonTam pharmaceuticals co. Zhongshan-China and distilled water were used as controls.

Determination of Minimum Inhibitory Concentration (MIC)

MICs were determined as described by NCCLS.[7] Crude extracts were twofold diluted in Mueller-Hinton broth for bacteria. A 100 µl of each dilution was dispensed in well of microdilution plates (96-wells). Well was inoculated with 50 µl of previously prepared standard culture of bacteria. The inoculated plates were incubated at 35ºC for 24 h and examined for visible growth in order to determine MIC. The previous controls were also included.

Table 1: Antibacterial activity of fresh parts of *V. radiata* on isolated bacteria by well agar diffusion method

Plant parts	Extract type	Concen. (mg/ml)	Zone of inhibition (mm)				
			S. aureus	*E. coli*	*K. pneumoniae*	*B. subtilis*	*P. vulgaris*
Leaves	Ethanolic	50	–	14*	–	19*	16*
		25	–	12*	–	18*	16*
		12.5	–	–	–	15*	14*
		6.25	–	–	–	13	–
		3.125	–	–	–	–	–
	aqueous	50	–	15*	–	17*	18*
		25	–	12*	–	16*	15*
		12.5	–	11	–	16*	11
		6.25	–	–	–	10	–
		3.125	–	–	–	–	–
Stem	Ethanolic	50	–	–	–	–	–
		25	–	–	–	–	–
		12.5	–	–	–	–	–
		6.25	–	–	–	–	–
		3.125	–	–	–	–	–
	Aqueous	50	15*	17*	–	20*	22*
		25	–	16*	–	20*	19*
		12.5	–	9	–	18*	15*
		6.25	–	–	–	13	11
		3.125	–	–	–	10	–
Root	Ethanolic	50	–	13*	–	–	–
		25	–	11	–	–	–
		12.5	–	–	–	–	–
		6.25	–	–	–	–	–
		3.125	–	–	–	–	–
	Aqueous	50	–	–	–	–	–
		25	–	–	–	–	–
		12.5	–	–	–	–	–
		6.25	–	–	–	–	–
		3.125	–	–	–	–	–
Cefotaxime		30 µg/ml	27	22	–	34	21

*** Significant differences ($P < 0.05$) between parts of plant and cefotaxime**

Statistical analysis

Results were statistically analyzed by using two-way variance of analysis (ANOVA) with less significant difference (L.S.D.) at $P < 0.05$.

RESULTS

Antibacterial activity of fresh and dried parts of *Vigna radiata* was investigated using standard well agar diffusion method. Data of this study revealed variable results of antibacterial effect of different plant parts. Extracts prepared from dried stem and root exhibited better antibacterial activities with significant differences ($P < 0.05$) from standard antibacterial agent (cefotaxime) than those prepared from fresh plant, while leaves extract showed reflective results. The extract of fresh leaves revealed more effective than of dried leaves. Otherwise, there is no significant differences ($P < 0.05$) was noted between the activity of ethanolic and aqueous extracts on all tested bacteria (Table 1 and 2).

Fresh aqueous extracts of root and aqueous and ethanolic extract of stem exhibited no activity on all isolated strains, while dried extracts of the same plant parts showed much more antibacterial effects.

Based on bacteria strain, *K. pneumoniae* showed resistance to all types of extracts of all plant parts, followed by *S. aureus* in most concentrations. On the other hand, *B. subtilis* as one of gram positive bacteria showed more susceptibility to most extracts, especially to dried extracts of stem and root with least MIC value (Table 3). Meanwhile, gram negative bacteria represented by *P. vulgaris* were also susceptible to most concentrations of plant parts, especially to dried extracts. Thus, both of gram positive and negative could be considered have the same susceptibility to *V. radiata* extracts.

DISCUSSION

Although large numbers of plants are constantly being screened for their antimicrobial effects, plant kingdom still holds many species containing substances of medicinal value that have yet to be discovered. *V. radiata* considers important plant enrichment with valuable contents including

Table 2: Antibacterial activity of dried parts of *V. radiata* on isolated bacteria by well agar diffusion method

Plant parts	Extract type	Concen. (mg/ml)	Zone of inhibition (mm)				
			S. aureus	*E. coli*	*K. pneumoniae*	*B. subtilis*	*P. vulgaris*
Leaves	Ethanolic	50	–	14*	–	20*	17*
		25	–	–	–	–	–
		12.5	–	–	–	–	–
		6.25	–	–	–	–	–
		3.125	–	–	–	–	–
	Aqueous	50	–	18*	–	18*	18
		25	–	11*	–	11	–
		12.5	–	–	–	–	–
		6.25	–	–	–	–	–
		3.125	–	–	–	–	–
Stem	Ethanolic	50	17*	16*	–	24*	19*
		25	–	14*	–	20*	17*
		12.5	–	13*	–	16*	14*
		6.25	–	11*	–	13	13*
		3.125	–	–	–	11	11
	Aqueous	50	15*	17*	–	20*	22*
		25	–	16*	–	20*	19*
		12.5	–	9	–	18*	15*
		6.25	–	–	–	13	11*
		3.125	–	–	–	10	–
Root	Ethanolic	50	18*	15*	–	23*	19*
		25	–	13*	–	21*	18*
		12.5	–	11*	–	19*	15*
		6.25	–	–	–	13	12*
		3.125	–	–	–	12	10
	Aqueous	50	–	11*	–	22*	20*
		25	–	10	–	20*	15*
		12.5	–	–	–	17*	10
		6.25	–	–	–	11	9
		3.125	–	–	–	–	–
Cefotaxime		30 µg/ml	27	22	–	34	21

*** Significant differences ($P < 0.05$) between parts of plant and cefotaxime**

Table 3: MICs (mg/ml) of *V. radiata* extracts in pathogenic bacteria

Plant parts	Extract type	*S. aureus*	*E. coli*	*B. subtilis*	*P. vulgaris*
Fresh leaves	Ethanolic	>50	16	4	10
	Aqueous	>50	8	4	10
Dried leaves	Ethanolic	>50	30	40	32
	Aqueous	>50	16	16	36
Fresh stem	Ethanolic	>50	>50	>50	>50
	Aqueous	>50	>50	>50	>50
Dried stem	Ethanolic	32	4	1	1
	Aqueous	38	10	0.8	4
Fresh root	Ethanolic	>50	16	>50	>50
	Aqueous	>50	>50	>50	>50
Dried root	Ethanolic	48	8	1.2	0.8
	Aqueous	>50	10	0.8	>50

proteins, arachidic acid, arginine, ascorbic acid, genstein and shikimic acid.[8] Other components that protect *V. radiata* from microbial infection may also present. Thus, exposure of *V. radiata* to pathogenic microorganisms stimulated production of antioxidant activity and phenolic compounds.[9]

Based on present study, it is well demonstrated that extracts prepared from dried plant parts revealed better antibacterial activity than those prepared from fresh parts. Extracts of dried stem and root showed greater diameter of zone of inhibition on cultured of most isolated bacteria. While, fresh extract of *Pelargonium radula*, in previous study, showed more effects on both gram positive and negative bacteria than dried extract.[2]

Data also indicated that both strains of gram positive and negative were affected by plant materials at the equal level with no difference in the susceptibility of these groups to each of plant extracts. *K. pneumoniae* as one strain of gram negative and *S. aureus* as one strain of gram positive showed more resistant to plant extracts. Meanwhile, *B. subtilis* (gram positive) and *P. vulgaris* (gram negative) revealed susceptibility to these extracts. The similarity in the response of two main groups of bacteria to plant extracts may related to the presence of broad spectrum compounds in *V. radiata* that active against gram positive and gram negative bacteria.

There is frequently much variable suggestion about which type of solvent is preferred to use in extraction method that can exhibit the potential activity of plant on bacteria. In some cases, ethanolic extract showed more efficiency to inhibit microorganism's growth as noted with the activity of ethanolic extracts of *Catharanthus rose* on six strains of bacteria.[4] Otherwise, two strains of *S. aureus* inhibited by aqueous extracts of V. *radiata*, while ethanolic extract effected on other strain (*S. subflava*).[8] Furthermore, dried alcoholic extract of *V. radiata* seeds was failed to inhibit the bacterium *Burkholderia pseudomallei*.[10] However, parts of *V. radiata* in the form of ethanolic and aqueous extracts revealed approximately the same manner of activity on isolated bacteria.

CONCLUSIONS

V. radiata has a potential antibacterial activity on clinically isolated bacteria. Dried extracts showed more effective action on tested bacteria than fresh extracts. Furthermore, no differences were noted between ethanolic and aqueous extracts on both of gram positive and negative bacteria with no differences in the susceptibility of each group of bacteria to any part of plant.

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In vivo and In vitro Antioxidant and Hepatoprotective effects of Classical ayurvedic formulation Punarnavashtak kwath against Ethanol induced hepatotoxicity

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ABSTRACT

Punarnavashtak kwath (PN) a classical Ayurvedic formulation reported in "Bhaishyajyaratnavali" consisting of eight medicinal plants was evaluated for its *in vitro* and *in vivo* antioxidant activity and hepatoprotective effect. *In vitro* antioxidant activity of PN kwath was investigated by DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity, superoxide anion and nitric oxide radical scavenging activity and reducing power assay. *In vitro* antioxidant activity was found to be dose dependent. Hepatoprotective and *in vivo* antioxidant effect was evaluated by ethanol (3.7 g/kg, p.o for 45 days in all group except control) induced hepatic damage in rats. Pretreatment with PN kwath 100 mg/kg p.o for 45 days) significantly prevented physical (increased liver wt), biochemical (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), bilirubin and total protein level), histological (damage to hepatocytes) and functional changes (thiopentone induced sleeping time) induced by ethanol in liver. Further PN kwath showed antioxidant activity by increasing activity of GSH, SOD and CAT and by decreasing the level of thiobarbituric acid reactive substance (TBARS). The results were compared to that of reference standard silymarin (50 mg/kg p.o for 45 days). The findings suggest that PN kwath protects the liver cell from ethanol induced liver damages due to its antioxidative effect on hepatocytes.

Key words: Ethanol, Hepatoprotective activity, *In vivo* antioxidant activity; *In vitro* antioxidant activity, Punarnavashtak kwath

INTRODUCTION

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals.[1] Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism.[2] The most common reactive oxygen species (ROS) include superoxide (0_2^-) anion, hydrogen peroxide (H_20_2), peroxyl (ROO^-) radicals and reactive hydroxyl (OH) radicals. The nitrogen derived free radicals are nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$). ROS have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome.[3] In treatment of these diseases, antioxidant therapy has gained an immense importance. Worldover the medicinal properties of plants have been investigated in the recent scientific developments, due to their potent antioxidant activities, no side effects and economic viability.[4] Flavonoids and phenolic compounds widely distributed in plants have been reported to exert multiple biological effects like antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic activity etc.[5] They were also speculated to be a potential iron chelators.[6-7]

Liver diseases remain a serious health problem. It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals.[8] Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India.[9]

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Alcohol dependency is a major health and socioeconomic problem throughout the world.[10-11] It has been observed that most of the consumed alcohol is eventually broken down by the liver and the products generated and accumulated during alcohol metabolism (e.g. acetaldehyde) are more toxic than alcohol itself. In addition, a group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body's natural defenses against free radicals (e.g. antioxidants) are inhibited by alcohol consumption, leading to increased liver damage.[12] Despite great progress made in the field in the past two decades, development of suitable medications for the treatment of alcohol dependency or alcohol-induced health injury remains a challenging goal for management/treatment of alcohol induced hepatotoxicity.

Traditional medicines are effective in certain disorders and are based on experience in the use of plant products in amelioration of common diseases. In the present investigation Punarnavashtak kwath, a classical Ayurvedic polyherbal (Table 1) formulation mentioned in Ayurveda,[13] consisting of *Boerhaavia diffusa* Linn, *Picrorhiza Kurroa* Royle ex Benth, *Tinospora cordifolia* (Willd.) Miers, *Zingiber officinalis* Rosc, *Berberis aristata* DC, *Terminalia chebula* Retz, *Azadirachta indica* A. Juss and *Tricosanthes dioica* Roxb. plants has been evaluated for its hepatoprotective action against ethanol induced hepatotoxicity. Traditionally this formulation is used in treatment of hepatic disorders and asthma. Many of the individual ingredients of the formulation are reported earlier for their protective activity against different models of experimental hepatotoxicity. An aqueous extract of thinner roots of *Boerhaavia diffusa* at a dose of 2 ml /kg exhibited marked protection of various enzymes such as SGOT, SGPT and bilirubin in serum against hepatic injury in rats.[14] The active constituents of *Picrorhiza Kurroa* were effective in preventing liver toxicity and the subsequent biochemical changes caused by numerous toxic agents.[15] Picrorhiza extract, when given at a dose of 3-12 mg/kg orally for 45 days, was also shown to be effective in reversing ethanol-induced liver damage in rats.[16] The hepatoprotective action of *Tinospora cordifolia* was reported in one of the experiment in which goats treated with *Tinospora cordifolia* have shown significant clinical and hemato-biochemical improvement in CCl_4 induced hepatopathy. Extract of *Tinospora cordifolia* has also exhibited *in vitro* inactivating property against Hepatitis B and E surface antigen in 48-72 h.[17] The aqueous ethanol extract of *Zingiber officinalis* showed hepatoprotective effect against acetaminophen-induced acute toxicity, mediated either by preventing the decline of hepatic antioxidant status or due to its direct radical scavenging capacity.[18] *Berberis aristata* and berberine (an alkaloid from *Berberis aristata*) were found to be protective against both paracetamol and CCl_4 induced liver damage and also showed MDME (microsomal drug metabolizing enzymes) inhibitory activities.[19] *Terminalia chebula* extract was found to prevent the hepatotoxicity caused by the administration of rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA) (in combination) in a sub-chronic mode.[20] The aqueous extract of *Azadirachta indica* leaf was found to offer protection against paracetamol induced liver necrosis in rats.[21] *Tricosanthes dioica* was reported as a hepatoprotective agent in ferrous sulphate ($FeSO_4$) intoxicated rats.[22] Poly herbal formulation have synergistic potentiative agonistic/ antagonistic agent within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects.

There is lack of scientific data regarding pharmacological evaluation of PN kwath, consequently it was considered worthwhile to screen PN kwath for its hepatoprotective activity. To understand the mechanisms of its pharmacological actions, *in vitro* and *in vivo* antioxidant activity of PN kwath was investigated.

MATERIALS AND METHODS

Collection of plants and Preparation of formulation

Punarnava, Galo, Tricosanthes, Neem were collected from medicinal garden of APMC College of Pharmaceutical Education and Research (January 2008) and other plants (Picrrorhiza, Berberis, Harde and Ginger) were collected from market. All the plants were authentified by the botanist, H.N.S.B Science College, Himatnagar and voucher specimen of all plants were kept in department of Pharmacognosy

Table 1: Composition of Punarnavashtak kwath.

No	Botanical name	Family	Part used
1	*Boer haavia diffusa* Linn.	Nyctaginaceae	Root
2	*Azadirachta indica* A. Juss.	Meliaceae	Bark
3	*Tricosanthes dioica* Roxb.	Cucurbitaceae	Whole
4	*Terminalia chebula* Retz,	Combretaceae	Fruit
5	*Zingiber officinalis* Rosc.	Zingiberaceae	Rhizome
6	*Tinospora cordifolia* (Willd.) Miers.	Menispermaceae	Stem
7	*Berberis aristata* DC.	Berberidaceae	Stem
8	*Picrorhiza Kurroa* Royle ex Benth	Scrophulariaceae	Root

APMC College of Pharmaceutical education and research. (APMC 0801 to 0808). Kwath (decoction) was prepared by boiling powder of all drugs (Table 1) in equal quantity in proportion of 16 times of water reduced to one fourth and strained in cloth.[13] Filtrate was evaporated and dried under reduced pressure. Yield of extract was 10% w/w.

Materials

Diagnostic kits were purchased from span diagnostic Ltd (Baroda, India) Reduced glutathione and thiobarbituric acid were purchased from Kemphasol (Mumbai, India). Tris-HCl was from Loba Chemie Pvt Ltd. (Mumbai, India). All other chemicals were obtained from SD fine chemicals. (Mumbai, India).

Preliminary phytochemical screening

The dried extract of kwath was subjected to the preliminary phytochemical analysis for the presence of different phytoconstituents.[23]

Acute toxicity study

Swiss albino mice of either sex weighing between 25-30 g were divided into ten groups of six animals in each.[24] The control group received normal saline (2 ml / kg, p.o). The other groups received 100, 200, 300, 600, 800, 1000, 2000, 3000, 5000 mg/kg of the test extract, respectively. Immediately after dosing, the animals were observed continuously for the first 4 h for any behavioral changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any. The observations were made twice daily, one at 7 a.m. and another at 7 p.m.

Preparation of stock solution for quantification of polyphenol and Flavonoids

Stock solution of PN kwath 1mg/ml in distilled water was prepared and used for estimation of polyphenol and flavonoids

Quantification of Polyphenol in PN Kwath

Total phenol content in plant extracts was generally determined according to the Folin-ciocalteu method. This colorimetric method is based on the reduction of a Phosphotungstate phosphomolybdate complex by phenolics to blue color products in alkaline conditions.

Each of the 100 µl of samples taken in to 25 ml volumetric flask, to which 10 ml of water and 1.5 ml of Folin Ciocalteau reagent were added. The mixture was then kept for 5 min. and to it 4 ml of 20% w/v sodium carbonate solution was added, the volume was made up to 25 ml with double distilled water. The mixture was kept for 30 minute until blue color developed. The samples were then observed at 765 nm in UV- visible spectrometer Shimadzu, UV-1601, Japan. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure.[25-27]

Quantification of total flavonoids

Aluminum chloride colorimetric method was used for flavonoids determination.[28] The method is based on the quantification of yellow color produced by the interaction of flavonoids with $AlCl_3$ reagent. 1 ml of sample from stock solution was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV Visible spectrophotometer. The % of total flavonoid was calculated by plotting calibration curve of standard flavonoid (Rutin).

In vitro antioxidant activity

Preparation of stock solution for in vitro antioxidant activity

Methanolic extract of PN kwath was taken and stock solution 1mg/ml was prepared. From the stock solution different concentration of solution was prepared.

DPPH free radical scavenging activity

4.3 mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150 µl DPPH solution was added to 3 ml methanol and absorbance was taken immediately at 516 nm for control reading. Different concentrations of kwath were taken and the volume was made uniformly to 150 µl using methanol. Each of the samples was then further diluted with methanol up to 3 ml and to each, 150 µl DPPH was added. Absorbance was taken after 15 min. at 516 nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value for kwath was calculated.[29]

Super Oxide free radical scavenging activity

100 µl Riboflavin solution [20 µg], 200 µl EDTA solution [12 mM], 200 µl methanol and 100 µl NBT (Nitro-blue tetrazolium) solution [0.1 mg] were mixed in test tube and reaction mixture was diluted up to 3 ml with phosphate buffer [50 mM]. The absorbance of solution was measured at 590 nm using phosphate buffer as blank after illumination for 5min. This is taken as control. Different concentrations of kwath were diluted up to 100 µl with methanol, to each of this, 100 µl Riboflavin, 200 µl EDTA, 200 µl methanol and 100 µl NBT were mixed in test tubes and further diluted up to 3 ml with phosphate buffer. Absorbance was measured after illumination for 5 min. at 590 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. IC_{50} value for kwath was calculated.[30-31]

Nitric Oxide scavenging activity

Different concentrations of the kwath were taken in separate tubes and the volume was uniformly made up to 150 µl with methanol. To each tube 2.0 ml, of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes. Similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV visible spectrometer Shimadzu, UV-1601, Japan. Curcumin was used as positive control.[32-34]

The DPPH free radical scavenging activity, super oxide free radical scavenging activity and nitric oxide scavenging activity were calculated using the following formula:

$$\%\,\text{Reduction} = \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100$$

Reducing power assay

The reducing power of phenolic samples was determined by the method of Jayaprakasha et al.[35] Different concentrations of kwath dissolved in methanol (1 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubation 20 min at 50°C, 2.5 ml of 10% trichloroacetic acid was added to the mixtures. After centrifugation, 2.5 ml of the upper layer was diluted with distilled water and 0.5 ml of 0.1% ferric chloride was added. The absorbance was measured at 700 nm. Increase in absorbance of the reaction indicated the reducing power of the polyphenol test samples.

Hepatoprotective and in vivo antioxidant activity

Healthy male albino Wistar rats 180-250 g were used. They were collected from animal house, Zydus Cadila Pharmaceuticals, Ahmedabad. The animals were grouped and housed in poly acrylic cages, with not more than two animal per cage and maintained under well-controlled conditions of temperature (27 ± 2°C), humidity (55 ± 5 %) and 12/12 h light-dark cycle were used for the study. Conventional laboratory diet and tap water were provided *ad libitum*. The protocol of the experiment was approved by the Institutional Animal Ethical Committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (Proposal no. 08/02, APMC, Himatnagar)

The animals were divided into four groups. Each group consisted of six rats. Normal rats were kept in group I. The rats of group II were given ethyl alcohol (3.7 g/kg) for 45 days.[36] Group III animals received simultaneous feeding of both ethyl alcohol (3.7 g/kg) and PN kwath (100 mg/kg p o) for 45 days. Group IV animals received simultaneous feeding of both ethyl alcohol (3.7 g/kg) and Silymarin (50 mg/kg p o) for 45 days. After last dose of PN kwath, thiopentone sodium was injected (40 mg/kg i.p) in all group to record sleeping time.[37]

Experimental analysis was carried out 24 h after the last dose of ethyl alcohol. Liver was isolated and used for antioxidant activity and histopathological analysis

Biochemical studies

Blood was taken from rats by puncture of the retro-orbital plexus, after 24 h of last dose of ethyl alcohol and allowed to coagulate at 37°C for 2 h, serum was separated by centrifugation at 3000 rpm for 10 min and analyzed for various biochemical parameter namely SGPT, SGOT,[38] SALP,[39] serum bilirubin,[40] and protein content.[41]

In vivo antioxidant activity

For estimating antioxidant activity, animals were sacrificed and their liver was excised, rinsed in ice-cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation.[42] A part of homogenate after precipitating proteins with trichloro acetic acid (TCA) was used for estimation of glutathione.[43] The remaining homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of super oxide dismutase[44] and Catalase.[45]

Histopathological studies

Paraffin sections (7 µm thick) of buffered formalin-fixed liver samples were stained with hematoxylin-eosin to study the histological structure of control and treated rats liver.[46]

Statistical analysis

Results are expressed as mean ± S.E.M. The statistical difference was analyzed by one way analysis of variance followed by Tukey-kramer multiple comparison test, significance was calculated as the P value, and P values of less than 0.05 were regarded as statistically significant.

RESULTS

Preliminary phytochemical studies revealed the presence of alkaloid, tannin, saponin, flavonoid and bitter principle in PN kwath.

In acute toxicity study, it was observed that there was no mortality at any of the tested doses (Up to 5000 mg/kg) till the end of 14 days of observation.

Total phenolic and Flavonoid Content

Total phenolic and flavonoids compound were found to be present 7.24 ± 0.037% and 2.95 ± 0.13% respectively in PN kwath

In vitro antioxidant activity

PN kwath possessed significant antioxidant activity in DPPH assay with IC_{50} value 14.97 µg/ml and was compared with the reference drug ascorbic acid (Table 2). PN kwath also possessed significant nitric oxide scavenging activity with IC_{50} value 97.10 µg/ml and superoxide free radical scavenging activity with IC_{50} value 6.40 µg/ml was compared with reference drug (Table 3 & 4). The reducing power of PN kwath was increased with increasing dosage (Figure 1) showed significant antioxidant activity.

Hepatoprotective activity

Treatment of rats with ethanol produced an increase in the weight of wet-liver. Rats pretreated with silymarin and PN kwath showed significant decrease in wet-liver weight compared to toxic group (Table 5). Hepatic damage induced by ethanol caused significant rise in marker enzymes SGPT, SGOT, ALP, bilirubin and decrease the level of protein. Oral administration of PN kwath was seen to lower significantly the levels of marker enzymes (SGPT, SGOT, ALP, and Bilirubin) and significantly increased the protein level in rats, compared to ethanol treated group (Table 6). A significant reduction in thiopentone induced sleeping time was observed with PN kwath treated animals compared to ethanol treated animals (Table 5). Effects of PN kwath were compared with standard reference drug silymarin.

In vivo antioxidant activity

The effect of PN kwath on lipid peroxidation (TBARS), glutathione, super oxide dismutase and catalase levels are

Table 2: Effect of PN kwath on DPPH free radical scavenging activity.

Conc (µg/ml)	% Inhibition	IC_{50} µg/ml	R^2
4	17.50 ± 2.51		
6	23.14 ± 1.40		
8	27.40 ± 1.37	14.97	0.995
10	34.70 ± 2.09		
15	50.68 ± 2.09		
Std (ascorbic acid)		8.75	0.9919

The sign R^2 is correlation of Regression
Values are means ± SD (n=3).

Table 3: Effect of PN kwath on Nitric oxide scavenging activity

Conc (µg/ml)	% Inhibition	IC_{50} µg/ml	R^2
20	10.74 ± 0.36		
40	21.40 ± 1.11		
60	33.29 ± 1.65	97.105	0.9947
80	40.22 ± 1.73		
100	51.42 ± 1.65		
Std (Curcumin)		17.72	0.9921

Values are means ± SD (n=3).
The sign R^2 is correlation of Regression.

Table 4: Effect of PN kwath on Super oxide free radical scavenging activity.

Conc (µg/ml)	% Inhibition	IC_{50} µg/ml	R^2
4	36.61 ± 1.49		
6	49.70 ± 0.30		
8	57.08 ± 0.52	6.40	0.990
10	69.46 ± 1.16		
15	88.99 ± 0.12		
Std (Ascorbic acid)		4.77	0.990

Values are means ± SD (n=3).
The sign R^2 is correlation of Regression.

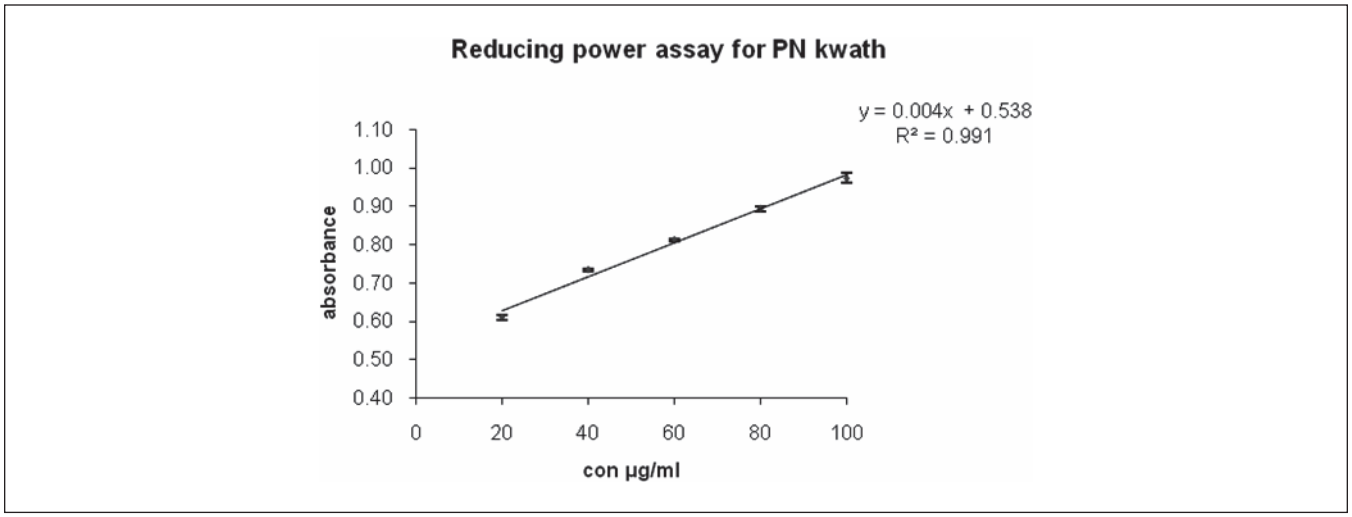

Figure 1: Reducing power of PN kwath at different concentrations.
Each value represents means ± SD (n=3). High absorbance at 700 nm indicates high reducing power.

shown in Table 7. Thiobarbituric acid reactive substance levels were significantly increased in the ethanol treated rats when compared with the normal rats. Treatment with PN kwath significantly prevented the increase in TBARS levels and brought them near to normal level. GSH and CAT activity were significantly increased in PN kwath treated groups. PN kwath also showed increase in activity of SOD compare to toxicant but it was not significant. The effects of PN kwath were comparable to that of standard reference drug silymarin.

Histopathological study

Hepatocytes of the normal control group showed a normal lobular architecture of the liver. In the ethanol treated group the liver showed microvascular fatty changes and the hepatocytes were surrounded by large number of fat droplets.(Figure 2a, Figure 2b) Silymarin and PN kwath pretreated groups showed minimal fatty changes and their lobular architecture was normal, indicating the hepatoprotective effect of these extracts (Figure 2c, 2d).

DISCUSSION

Although oxygen is essential for life, its transformation to reactive oxygen species (ROS) may provoke uncontrolled reactions. Such challenges may arise due to exposure to radiation, chemicals or by other means. Antioxidants may offer resistance against oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and some other mechanism.[7]

Polyphenol and flavonoids are used for the prevention and cure of various diseases which is mainly associated with free

Table 5: Effect of Punarnavashtak kwath on different biochemical parameter in ethanol induced hepatotoxicity.

Group	SGOT(U/L)	SGPT(U/L)	ALKP (U/L)	Bilirubin(mg%)	Protein(mg/dl)
I (control)	40.33 ± 2.26	103.83 ± 7.31	130.17 ± 8.50	0.56 ± 0.03	5.65 ± 0.27
II (Toxicant)	151.17 ± 4.34[a]	280.83 ± 9.51[a]	288.50 ± 13.44[a]	1.18 ± 0.09[a]	3.88 ± 0.14[a]
III (PN kwath)	94.67 ± 3.16***	157.67 ± 10.58***	186.83 ± 13.59***	0.80 ±0.04**	5.23 ± 0.30**
IV (Silymarin)	77.50 ± 6.67**	128.67 ± 10.66***	157.33 ± 10..3***	0.66 ± 0.06***	5.42 ± 0.26**

Values are mean ± SEM of 6 animals in each group
[a]P < 0.001 relative to control group.
***P < 0.001 relative to Toxicant group.
**P < 0.01 relative to Toxicant group.

Table 6: Effect of Punarnavashtak kwath on thiopentone induced sleeping time and weight of liver.

Group	Thiopentone sodium induced sleeping time		Liver wt gm/100gm bw
	Onset(s)	Duration(min)	
I (control)	202.50 ± 4.96	76.67 ± 4.94	3.95 ± 0.51
II (Toxicant)	53.33 ± 4.22[a]	243.33 ± 4.77[a]	6.42 ± 0.32[a]
III (PN kwath)	163.50 ± 7.25***	132.50 ± 8.83***	5.25 ± 0.16*
IV (Silymarin)	179.17 ± 9.44***	120.63 ± 6.64***	4.83 ± 0.14*

Values are mean ± SEM of 6 animals in each group
[a]P < 0.001 relative to control group
***P < 0.001 relative to Toxicant group
*P < 0.05 relative to Toxicant group

Table 7: Effect of Punarnavashtak on lipid peroxidation (MDA), superoxide dismutase (SOD), catalase and glutathione level in ethanol induced hepatotoxicity

Group	TBRAS	Gutathione	Catalase	SOD
	nmol/ mg protein	µg/mg protein	u/min/mgprotein	U/mgprotein
I (control)	1.92 ± 0.25	56.85 ± 4.26	36.36 ± 4.82	14.59 ± 1.76
II (Toxicant)	7.23 ± 0.70[a]	31.87 ± 6.44[b]	9.12 ± 1.65[a]	4.02 ± 0.62[a]
III (PN kwath)	3.14 ± 0.44***	39.48 ± 3.61*	23.75 ± 0.81*	7.87 ± 0.65*
IV (Silymarin)	2.49 ± 0.41***	46.75 ± 7.80*	25.02 ± 3.10*	8.03 ± 0.63*

Values are mean ± SEM of 6 animals in each group
[a] P < 0.001 relative to control group
[b] P < 0.01 relative to control group
****P < 0.001 relative to Toxicant group*
*** P < 0.05 relative to Toxicant group*

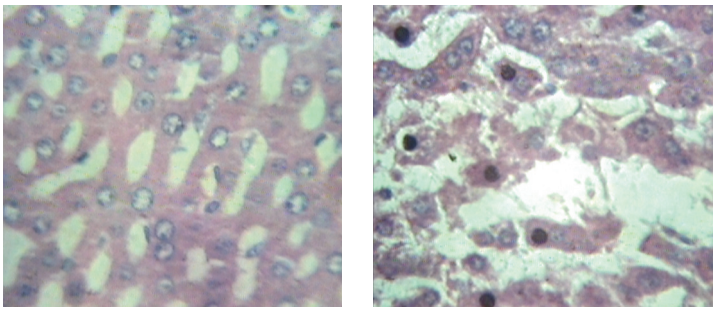

Figure 2A: Control

Figure 2B: Toxicant

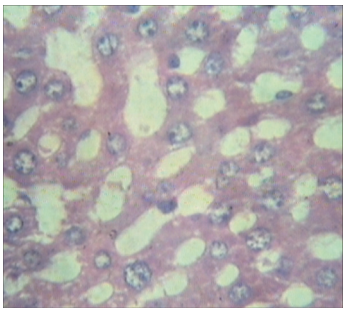

Figure 2C: PN Kwath

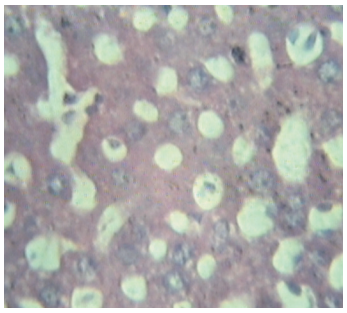

Figure 2D: Silymarine

Figure 2a: Normal control rat showed hepatic cells with brought out nuclei, cytoplasm, central vein and portal triad. Figure 2b: Ethanol-treated group showed micro vascular fatty changes and the hepatocytes were surrounded by large number of fat droplates. Figure 2c and Figure 2d: PN kwath and silymarin pretreated groups showed minimal fatty changes and their lobular architecture was normal, indicating the hepatoprotective effect of these extracts.

Figure 2: Photomicrograph of liver section taken from rats against ethanol induced hepatotoxicity (X45)

radicals. Significant amount of polyphenolic and flavonoid compound were present in PN kwath, which attributes to its rationality of possessing antioxidant activity.

DPPH radical is considered to be a model of lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid autooxidation. The radical scavenging activity of PN kwath was determined from the reduction in absorbance at 517 nm due to scavenging of stable DPPH free radical. The dose dependent inhibition of DPPH radical indicated that PN kwath causes reduction of DPPH radical in a stoichometric manner.[47]

Nitric oxide (NO) exhibits numerous physiological properties and it is also implicated in several pathological states.[48] However, the specificity of this assay has been questioned since nitrite is one final product of the reaction of nitric oxide with oxygen, through intermediates such as NO_3, N_2O_4 and N_2O_3.[49] Therefore the decrease in the nitrite production could also be due to interaction of the extract with other nitrogen oxides.[50]

The *in vitro* superoxide radical scavenging activity is measured by riboflavin/ light/ NBT (Nitroblue tetrazoline) system reduction. The method is based on generation of superoxide radicals by autooxidation of riboflavin in the presence of light. The superoxide radical reduces NBT to a blue colored formazone that can be measured at 560 nm. The capacity of the PN kwath to inhibit the colour to 50% is measured in terms of IC_{50}. Superoxide radical is known to be very harmful to the cellular components as a precursor of more ROS.[31] The extract has been found to have significant superoxide radical scavenging activity, which ultimately adds to its antioxidant potential.

For the measurements of the reducing ability, the $Fe_3^+$to Fe_2^+ transformation was investigated in the presence of PN kwath. The reducing power of PN kwath increased with increasing dosage.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[51-53]

Liver can be injured by many chemicals and drugs. In the present study ethanol was selected as a hepatotoxicant to induce liver damage, since it is clinically relevant. Ethanol produces a constellation of dose-related deleterious effects in the liver.[54] In chronic alcoholics, hepatomegaly occurs due to accumulation of lipids and proteins in hepatocytes,[55] with an impaired protein secretion by hepatocytes.[56] Water is retained in the cytoplasm of hepatocytes leading to enlargement of liver cells, resulting in increased total liver mass and volume[57] as observed in the present study. This alcohol-induced increase in total wet-liver weight was prevented by pretreatment with PN kwath, thus indicating a hepatoprotective effect.

The hepatoprotective activity of the ethanolic extract was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which are indicators of the functional state of the liver.[58] The increase in the levels of serum bilirubin reflected the degree of jaundice, while increase in hepatic enzymes indicate cellular leakage and loss of functional integrity of cell membrane.[59] It has been found that PN kwath effectively prevents ethanol-induced biochemical changes of liver toxicity.

Ethanol also alters the metabolic activity of hepatocytes, thereby inducing hepatic damage. Barbiturates are a class of xenobiotics that are extensively metabolized in the liver. Deranged liver function leads to delay in the clearance of barbiturates, resulting in a longer duration of hypnotic effect.[60] In the present study, administration of thiopentone sodium to rats pretreated chronically with alcohol resulted in an increased duration of thiopentone sleep time. Pre-treatment with PN kwath decreased thiopentone-induced sleep time, an indirect evidence of their hepatoprotective effect.

Formation of ROS, oxidative stress and hepatocellular injury has been implicated in alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic ethanol consumption, and these are primed and activated for enhanced formation of pro-inflammatory factors.[61] Additionally, alcohol-induced liver injury has been associated with increased amount of TBARS.[62] Indeed, PN kwath supplementation in our study was potentially effective in blunting TBARS, suggesting that PN kwath possibly has antioxidant property to reduce ethanol-induced membrane lipid peroxidation and thereby preserve membrane structure. It may thus be plausible that in our study, loss of membrane structure and integrity because of lipid peroxidation was accompanied with an elevated level of activities of SGOT, SGPT, ALP and bilirubin.

Our study further revealed that decrease in the activity of antioxidant enzymes SOD, CAT and GSH following ethanol exposure may be due to the damaging effects of free radicals, or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes. In our studies, it reveals that PN kwath could restore the activity of both these antioxidant enzymes and possibly could reduce generation of free radicals and hepatocellular damage.

Histological changes such as steatosis (fatty changes in hepatocytes) and perivenular fibrosis were observed in ethanol-treated (toxic) control group. Both the extracts prevented these histological changes, further indicating their hepatoprotective activity. All the histological changes observed were in correlation with the physical, biochemical, and functional parameters of the liver.

Ethanol, even after short-term consumption, induces CYP2E1 enzyme activity in doses that do not cause fatty changes. This enzyme accelerates alcohol metabolism with a resultant increase in acetaldehyde production.[63] Acetaldehyde is thought to have a number of adverse effects like decreased transport and secretion of proteins owing to tubulin polymerization, enhanced vitamin metabolism and trace metals and drugs like paracetamol cause severe acute toxicity which is sometimes fatal.[64-65]

Antioxidants exhibit hepatoprotective activity by blocking the conversion of ethanol to acetaldehyde.[66] From the above studies it was found that PN kwath exhibited an *in vitro* antioxidant property.[67,68] which may be responsible for the hepatoprotective activity of PN kwath. The presence of secondary metabolites like tannin, alkaloids, flavonoids, saponins and bitter principle in PN kwath may be responsible for the significant hepatoprotective activity. Detailed studies on the mechanism of action and phytochemical analysis are in progress at our laboratory.

ACKNOWLEDGEMENTS

The author express their sincere thanks to Dr. Ghanshyam R Patel, M.D. Ayurveda, B.A.P.S Swaminarayan Herbal Care, Shahibaug, Ahmedabad and Dr Tapan Vaidya, MD Ayurveda, Shri Dardi Narayan Sevamandal Hospital, Paldi, Ahmedabad, India for their generous help.

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Anti-ulcer activity of *cassia auriculata* leaf extract

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ABSTRACT

The present study was carried out to evaluate the anti-ulcer activity of *cassia auriculata* leaf extract against pylorous ligation induced gastric ulcer. The methanolic leaf extract of cassia *auriculata* at dose of 300 mg/kg p.o. markedly decrease the incidence of ulcers in pyloric ligatied rats. In pyloric ligated rats, there was an increase in the gastric volume, free and total acidity and ulcerative index as compared to the control group. The methanolic leaf extract of *cassia auriculata* at dose of, 300 mg/kg showed significant reduction in the above parameters which was comparable to the standard drug famotidine(10 mg/kg). *Cassia auriculata* extract showed protection index 79.4 %, whereas standard drug famotidine showed protection index 90.7%.

Key words: *Cassia auriculata*, Methanolic extract, Anti-ulceractivity, Pylorous ligation induced gastric ulcer and Famotidine

INTRODUCTION

Peptic ulcer disease (PUD) is a spectrum of diseases consisting of gastritis, gastric ulcers, and duodenal ulcers.[1] It is known to occur when the endogenous defense mechanisms of the protective mucosal barrier have failed to sufficiently counteract the aggressive factors (hydrochloric acid, pepsin, and *Helicobacter pyroli*) and is characterized by gnawing or burning sensation in the abdomen.[2] These agents have been implicated in the pathogenesis of gastric ulcer, including enhanced gastric acid and pepsin secretion, inhibition of prostaglandin synthesis and cell proliferation growth, diminished gastric blood flow and gastric motility.[3] Duodenal ulcers occurs more frequently (about 80% of PUDs) than gastric ulcers.

Medicinal herbs are significant source of pharmaceutical drug. Latest trends have shown increasing demand of phytodrugs and some medicinal herbs have been proven antiulcer activity .This paper describes the study of *Cassia auriculata* L. (Cesalpinaceae, common name: Tanner's Cassia) a common plant in Asia, has been widely used in traditional medicine as a cure for rheumatism, conjunctivitis and diabetes.[4] In addition, *Cassia auriculata* has been widely used in Ayurvedic medicine as 'Avarai Panchaga Choornam' and the main constituent of Kalpa herbal tea, has come under extensive study in the light of its antidiabetic effects. Recently had reported the antiperoxidative effect of *Cassia auriculata* flowers in streptozotocin diabetic rats.[5] The antidiabetic activity of aqueous extract of *C. auriculata* flowers has been documented previously.[6] The present study is an attempt to test the antiulcer activity of the *cassia auriculata* leaf extract.

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MATERIALS AND METHOD

Plant collection and identification

The basic plant material of *cassia auriculata* leaves used for the investigation was obtain from Mount Opera Garden, Near Ramoji Film City, Nalgonda dist,Andhra Pradesh, India. The plant can be identified and authenticated by department of Botany research office P. SUJATHA (Botanist), HOD, Bhavans New Science Degree College, Narayanaguda, Hyderabad.

Preparation of methanolic extract

The leaves were collected and shadow dried. The shade leaves were subjected to pulverization to get coarse powder. The coarsely powder leaves of *cassia auriculata* were used for extraction.

The shade dry coarsely leaves of *Cassia auriculata* were used for extraction with methanol. *Cassia auriculata* leaf powder (250 g) was loosely packed in the thimble of soxhlet apparatus and extracted with methanol at 55°C for 18 h. The extract was air dried at 25-30°C and weighed. For oral administration,

extract was dissolved in 10 mL Phosphate Buffer Saline (PBS) at different concentrations. To make the extract soluble in PBS, 1% tween 80 was used.

EXPERIMENTAL PROTOCOL

Experimental Animals

Wistar albino rats (150-200 g) of both sexes were obtained from the animal house of NIZAM INSTITUTE OF PHARMACY, Deshmukhi, Ramoji film city, Hyderabad. Before and during the experiment, rats were fed with standard diet (Gold Moher, Lipton India Ltd). After randomization into various groups and before initiation of experiment, the rats were acclimatized for a period of 7 days under standard environmental conditions of temperature, relative humidity, and dark/light cycle. Animals described as fasting were deprived of food and water for 16 h ad libitum. All animal experiment were carried out in accordance with the guidelines of CPCSEA and study was approved by the IAEC (Institutional animal ethical committee) with registeration number. (1330/AC/10/CPCSEA)

Acute Oral Toxicity Studies

Cassia auriculata at the dose range of 100 mg-2000 mg/kg were administered orally to different group of rats comprising of ten rats in each group. Mortality was observed after 72 h. Acute toxicity was determined according to the method of Litchfield and Wilcoxon.

ANTIULCER ACTIVITY

Experimental design for pyloric ligation induced gastric ulcer

Animals are divided into 4 groups, each comprising 6 rats.

Group I: Control group (without pyloric ligation).
Group II: Pyloric ligation for the induction of ulcers.
Group III: Pyloric ligated group + Extract (300 mg/kg) 1 hr before ligation
Group IV: Pyloric ligated group + Standard drug (Famotidine 10 mg/kg)

Methanolic leaf extract (300 mg/kg) was administered for a period of 7 days. On the 7th day normal saline, famotidine and methanolic extact of leaf were administered 1hr prior to pyloric ligation. Animals were anaesthetized using diethyl ether and the abdomen was opened and pylorus was ligated without causing any damage to its blood vassels. The stomach was replaced carefully and the abdominal wall was closed with interrupted sutures.[7,8] After 4hr of ligation, the animals were sacrificed by cervical dislocation. The abdomen was opened and a ligature was placed around the cardiac sphincter. The stomach was removed.[9] Gastric volume, free and total acid content of gastric juices were determined. Mean ulcer score for each animal was expressed as ulcerative index and the percentage ulcer protection was also calculated.

Estimation of gastric volume and free and total activity changes in pl model

Gastric volume

Four hours of ligation, stomachs were centrifuged and subjected to titration for estimation of free and total acid. One millimeter of the supernatant liquid was pipette out and diluted to 10 ml with distilled water. The solution was titrated against 0.01N NaOH using topfer's reagent as indicator, to endpoint when the solution turned to orange colouyr. The volume of NaOH needed was taken as corresponding to free acidity. Titration was further continued by adding 1% solution of phenolphthalein till the solution gained pink colour. The volume of NaOH required was noted and was taken as corresponding to total acidity. The sum of two titrations was total acidity.[10] Acidity was expressed as:

$$\text{Acidity} = \frac{\text{volume of NaOH} \times \text{normality} \times 100\ \text{mEq/L}/100\ \text{g}}{0.1}$$

Estimation of gastric ulcerative index changes in PL and model:

Ulcerative index was measured by method of takagi et al,[11] Briefly, the stomach was opened along the greater curvature. The stomach was washed with running tap water. Then it was placed on a flat wooden plate to count the ulcerative area.

The ulcer index was determined using the formula:

$$\text{Ulcer index} = \frac{10}{X}$$

Where, X = total mucosal area/total ulcerated area
Percentage ulcer protection was calculated using the formula:

$$\text{Ulcer protection (\%)} = \frac{100 - Ut}{Uc \times 100}$$

Where:

Ut = Ulcer index of treated group

Uc = Ulcer index of control group

STATISTICAL ANALYSIS

All the biochemical results were expressed as mean ± standard error of means(SEM). Data were analysed by tukey's multiple range tests using sigma stat version-3.5 software. A probability value of $p < 0.05$ was considersd to be statistically significant.

RESULTS

Experimental Results

Phytochemical screening of *Cassia auriculata* .of leaf extract shows the presence of Alkaloids, Flavonoids, Tannins, Carbohydrate, Glycosides and Saponin .The acute oral toxicity study of *Cassia auriculata* showed no mortality upto 2000 mg/kg. (**Table 1).**The antiulcer activity of *Cassia auriculata* is shown in **Table 2 and Table 3.** In pyloric ligated rats, there was an increase in the gastric volume, free and total acidity and ulcerative index as compared to the control group, extract showed reduction in gastric secretion free and total acidity and ulcerative index, at dose of, 300 mg/kg.(**Table 2 and Table 3**) showed significant reduction in the above parameters which was comparable to the standard drug famotidine.

Table 1. Acute toxicity studies of *Cassia auriculata*

S.no	Group	Dose	Wt of animal (gm)		Sing of toxicity	Onset of toxicity	Reversible or irreversible	Duration
			Before treatment (1st day)	after treatment (4th day)				
1	*Cassia auriculata*	2gm/kg	155	157	No Sign of toxicity	Nil	Nil	3 days
2	*Cassia auriculata*	2gm/kg	159	162	No Sign of toxicity	Nil	Nil	3 days
3	*Cassia auriculata*	2gm/kg	165	166	No Sign of toxicity	Nil	Nil	3 days
4	*Cassia auriculata*	2gm/kg	153	155	No Sign of toxicity	Nil	Nil	3 days
5	*Cassia auriculata*	2gm/kg	170	171	No Sign of toxicity	Nil	Nil	3 days
6	*Cassia auriculata*	2gm/kg	173	174	No Sign of toxicity	Nil	Nil	3 days

Table 2: Effect of extract on gastric secretion, free acidity, total acidity in pyloric ligated rats

Treatment	Gastric volume (mL/100 g)	Free acidity (mEq/100 g)	Total acidity (mEq/100 g)
Group I	1.2 ± 0.6	24.1 ± 0.94	60.2 ± 2.06
Group II	8.65 ± 0.2	63.5 ± 1.99	102.2 ± 1.38
Group III	3.5 ± 0.5	32.3 ± 1.42	73.6 ± 1.65
Group IV	2.4 ± 0.3	27.8 ± 1.44	61.7 ± 1.81

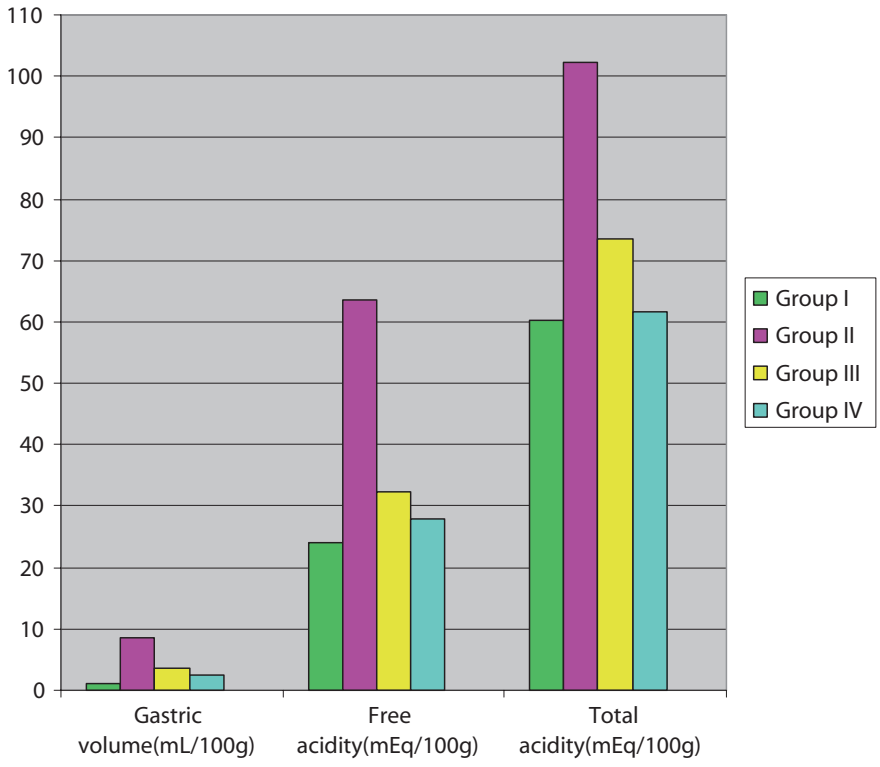
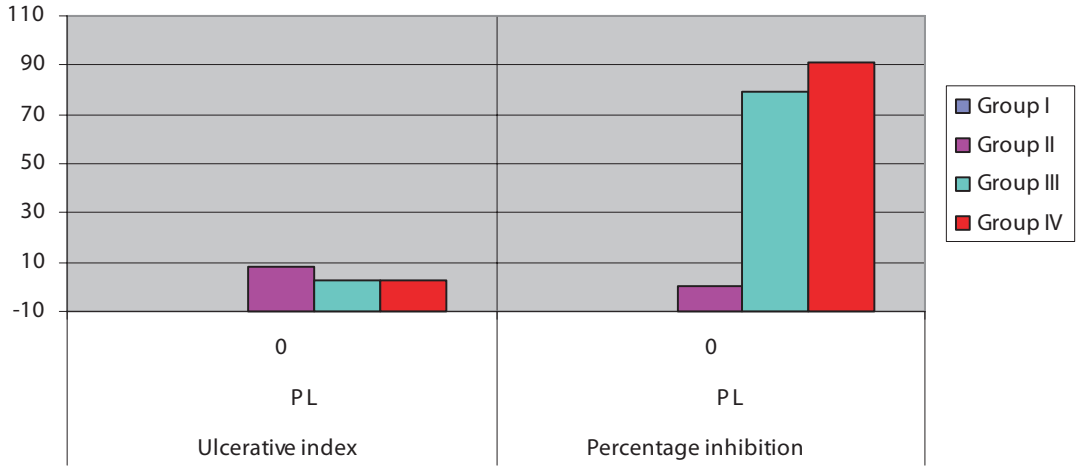


Table 3: Effect of MECS on ulcerative index and percentage inhibition in pl and wis induced gastric ulcer in rats

Groups (mg/kg)	Ulcerative index PL	Percentage inhibition PL
Group I	0.00 ± 0.00	0.0
Group II	8.1 ± 0.01	0.0
Group III	2.8 ± 0.01	79.4
Group IV	2.3 ± 0.01	90.7



DISCUSSION

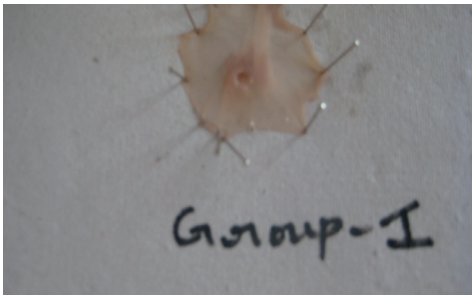
Various mechanisms are thought to be involved in the ulcer production in different experimental models.[12, 13] Hence it is not possible to produce a single mechanism for antiulcer effect of a particular drug. Digestive effect of the accumulated gastric juice is believed to be responsible for producing ulcers in pyloric ligated rats. In addition to gastric acid secretion, reflex neurogenic effect has also been suggested to important role in the formation of gastric ulcers in this model.[13, 14] Ulcer index parameter was used for the evaluation of anti ulcer activity since ulcer formation is directly related to factors such as gastric volume, free and total acidity.[15]

It is generally accepted that gastric ulcers result from an imbalance between aggressive factors and the maintenance of the mucosal integrity through endogenous defence mechanisms.[16] The excess gastric acid formation by prostaglandin (PG) includes both increase in mucosal resistance as well as a decrease in aggressive factors, mainly acid and pepsin.[17] Inhibitions of PG synthesis by aspirin coincide with the earlier stages of damage to the cell membrane of mucosal, parietal and endothelial cells.[18] To regain the balance, different therapeutic agents are used to inhibit the gastric acid secretion or to boost the mucosal defence mechanisms by increasing mucosal production, stabilising the surface epithelial cells or interfering with the prostaglandin synthesis. The causes of gastric ulcer pyloric ligation are believed to be due to stress induced increase in gastric hydrochloric acid secretion and/or stasis of acid and the volume of secretion is also an important factor in the formation of ulcer due to exposure of the unprotected lumen of the stomach to the accumulating acid.[19] The ligation of the pyloric end of the stomach causes accumulation of gastric acid in the stomach. This increase in the gastric acid secretion causes ulcers in the stomach.

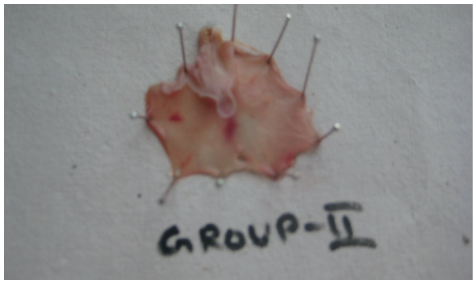
It is well known that free radicals are involved in the progression of ulcers. Pyloric ligated models increase in the oxidative and decrease in the antioxidative biomarkers have been reported.[20,21] Hence, as our results indicated maximum *in vitro* free radical scavenging activity along with ameliorative effect on various ulcerative parameters of *Cassia auriculata* extract so, this antioxidant potential may be responsible for its anti-ulcerogenic activity. Further studies are needed for their exact mechanism of action on gastric acid secretion and gastric cytoprotection.

CONCLUSION

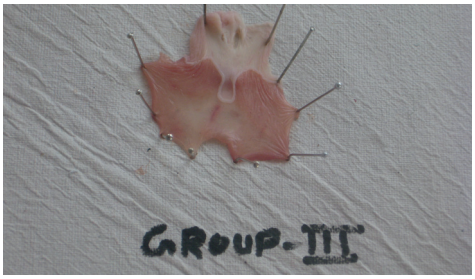
From the results obtained, it was observed that, the extract shows decrease in percent of incidence of ulcer and ulcer index in a dose dependent manner when compared with control group (Figure 1). After calculating the healing index (percent improvement), there was an increase in healing index in dose dependent manner when compared with



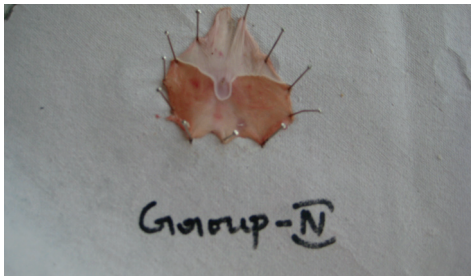
GROUP 1: Stomach of normal rat



GROUP II: Stomach of an ulcer control rat



GROUPIII: Stomach of a rat treated with 300 mg kg-1 **C. auriculataa** leaf methanolic extract



GROUP IV: Stomach of a rat treated with standerd drug famotidine (10 mg kg-1)

Figure 1

control group. So it was considered that the plant *cassia auriculata* has significantly decreased the no of ulcers in pyloric lygation induced gastric ulcers in rats. Thus the result from our study suggested that the plant *cassia auriculata* has the potent anti-ulcer property.

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Production of crude Drugs in Unani System of Medicine

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ABSTRACT

The crude drugs are being use since ADAM & EVE'S era. Descorides (100 A.D.), who is founder of Unani Pharnmacognosy (Ilmul Advia), He defined many ethics for production of crude drugs in Unani System of Medicine (USM), later many scholars of USM revealed the various methods. The crude drugs on reaching the pharmaceutical manufacturing line will have to pass through various stages, all of which influence the nature and amount of active constituents present. Its environmental conditions, cultivations, collections, drying and storage are the various aspects in usage of drugs.

Key words: Unani system of medicine, environmental condition, cultivation.

INTRODUCTION

In this modern age, people use everything in standard quality and that things would be more beneficial for their health, and least toxic/injurious to their health. Like water, it should be soft, purified, without contamination of bacteria, protozoa, and virus and even no excessive minerals and salts. Like this when human beings reside in a house, the atmosphere should not polluted and contaminated.

If we need to cure a patient, then we have to apply two main methods:

1. Diagnosis of a disease.
2. Treatment of a disease.

In Unani system of medicine, we are healing the patient by following three methods:

1. Elaj Bil Tadbeer/Regimental therapy
2. Elaj Bil Aghzia wal Advia/Diet and Drug Therapy.
3. Elaj Bil Yad/Surgery.[1]

Production of crude drugs in Unani System of Medicine, which comprises the methods of production of drugs and diets, which can used for treatment of patients. Veteran Unani Hakeems mentioned the criteria to produce a drug or diet that would be beneficial for human beings. if a Hakeem diagnose the patient and prescribe a single or compound drug formulations but the drug production could not be done properly, then that drug will not cure that disease, because that drug will not potential and his active ingredient will lesser or no active ingredient. Moreover, may be that drugs effect will be injurious instead of cure. So every Hakeem mentioned the criteria and methods to produce the drugs, but unlikely in this modern age both the Hakeem and pharmaceutical companies are not following the rules and methods of Unani reference Hakeem to control the quality of Unani drugs for better response and quality management.

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ENVIRONMENTAL CONDITIONS

1. Metabolites affected by temperature, rainfall, length of day (including the quality of light) and altitude. Such effects have been studied by growing particular plants in different climatic areas and observing variations.
2. Temperature is a major factor controlling the development and metabolism of plants. Although each species has become adapted to its own natural environment, plants are frequently able to exist in a considerable range of environment.
3. Certain drugs now obtained almost exclusively from cultivated plants. These include cardamoms, Indian hemp, ginger, peppermint and spearmint for oil production. Other includes Ceylon cinnamon, linseed, fennel, cinchona and opium. In other cases, both wild and cultivated plants are used. some plants have been cultivated sometimes immemorial (opium poppy and coca). Others are now grown because supplies of the

wild plants are insufficient to meet the demand are because owing to sparse distribution or inaccessibility, collection is difficult. Cultivation is essential in the case of the drugs Indian hemp & opium, which are subject to government control and recently for those plants in danger of over exploitation. In many cases, cultivation is advisable because of the improved quality of the drugs, which it is possible to produce. The improvement may be due to following…,

i. The power to confine collections to species, varieties or hybrids which have desired phytochemical characters (e.g. Aconite, Cinnamom, Cinchona)
ii. The better development of the plants owing to improve conditions of the soil, pruning and the control of the insects, pests, fungi etc.
iii. The better facilities for treatment after collection are drying at a correct temperature in the cases of digitalis, colchicum, belladonna and valerian and peeling of cinnamon and ginger.

For success in cultivation, it is necessary to study the conditions under which the plant flourishes in the wild state and reproduce the conditions are improving on them. Small changes in ecology can affect plant products.[2,3]

COLLECTION

Drugs are of vegetable (botanical), mineral and animal origin.[4]

Vegetable drugs

The vegetable drugs comprises of leaves, roots, seeds, branches, flowers, fruits, gums and all other plant parts.

a. **Leaves:** They should be pluck when they have attained their full size. They should be maintaining their form and color and their potency should not have diminished. Moreover, fallen and scattered leaves should not be taken.
b. **Branches:** they should take when they have reached perfection and have not started drying or crooking.
c. **Flowers:** they should be plucked when they have reached full bloom but have not dried up or fallen down.
d. **Fruits:** it is essential that they should be plucked when fully matured but before they fall down.

Overall, it is essential that the drugs to be procured should be fresh and seeds should have formed with in them, their roots should not be deformed or crooked, they should be mature and unshrivelled or broken are of no advantage; better among them are those, which have attained full size and weight. Nuts that are shriveled and broken are of no advantage; better among them are those, which have acquired their full weight. The drugs procured when the weather is clear better than those are, collected when the weather is humid or the rainy season is near. The drugs growing wild are stronger than those are cultivated; the former are generally smaller. The drugs growing on hills or mountains are stronger than those growing on plains are weaker. Drugs collected from forests and places that exposed to the sunrays, are better than those are collected at inappropriate times and from shady places. These guidelines should, however be followed as for as possible depending on the prevailing conditions. All those drugs, which have deep color, definite taste and distinct smell, are stronger. The strength of herbs is weak after three years. However, some drugs are exempted from this rule. E.g. the hellebores (black and white) which maintain their strength longer.[5]

e. **Seeds:** They should be procured when their substance has condensed and their rawness and moisture have disappeared.
f. **Gums:** They should be collected when coagulated but they grow not so much hard that they begin to be frittered away. Most of the gums, especially the farbium lose their strength after three years. The potency or strength of drugs depends upon its excellence. If it is difficult to get fresh drug of full strength, the older and weak may be taken in twice the amount of the fresh drug irrespective of its class.
g. **Roots:** They should be extracted when the trees have shed their leaves.

Animal drugs

They should be chosen from the young animals trapped during the rabi (spring season), should be of complete body and properly built, with all organs intact and discarding all such parts which after slaughtering and purifying are normal discarded. Animals that have died of some disease should not consider. These are the general principals which a physician ought to know with regard to simple drugs.[6]

Mineral drugs

Among the mineral drugs, the best are those, which are extracted from reputed mines; for example, green vitriol of Qabrus (Cyprus) and vitriol of Kirman, and those, which are free from adulteration. It is essential that the drug to be selected should be possessed its specific physical structure and maintaining its characteristics color and taste.

DRYING

If enzymatic action is to be encouraged, slow drying at a moderate temperature is necessary. Examples of this will be finding under 'Orriss Rhizome' Vanilla pods, Coca seeds

and Gentian root. If enzymatic action is not desired, drying should take place as soon as possible after collection. Drug containing volatile oils are liable to lose their aroma if not dried or if the oils are not distil from them immediately and all moist drugs are liable to develop mould. For these reasons, drying apparatus and distillation plants should situate as near to the growing plants. This has further advantage that freightage is much reduced, as many fresh drugs contain a considerable amount (60-90%) of water. The duration of the drying process varies from a few hours to many weeks, and in the case of open-air drying, depends very largely on the weather. In suitable climates, open-air drying is use for such drugs as clove, colocynth, cardamom and cinnamon. Even in warm and dry climates, arrangements have made for getting the drug under the cover of sheds or tarpaulins at night or during wet weather. For drying in sheds the drugs may be suspended in boundless from the roof, threaded on strings as in the case of Chinese rhubarb, or more commonly place on trace made of sacking of tinned wire–netting. Papers spread on a wooden framework are use, particularly for fruits form, which it is desired to collect the seeds.[7]

STORAGE

The large-scale storage of drugs is a considerable undertaking. Except in a few cases, such as cascara bark, long storage, although often unavoidable, is not to be recommended. Drugs such as Indian hemp and sarsaparilla deteriorate even when carefully stored. It has also reported that the content of taxol in taxu baccata leaves and extracts storage in a freezer and out of direct sunlight produce no adverse deterioration. Assuming that herbal drugs are going to be use medicinally, the same care will taken regarding their storage and packaging as with any other medicine. There are not many indications to be finding in the pharmacopoeia as how drugs should be stored. Important factors to which attention should be paid in regard to the storage of drugs and to how they should be kept.e.g. By patients, include light, temperature, humidity, degree of comminuting. Nearly all drugs require protection against light and this is specifically directed; this requirement arises, on the one hand, form the circumstance that leaf, flower, and herb drugs rapidly fade in light and become poor looking and, on the other hand, light accelerates numerous chemical processes, which may bring about degradation of or changes in the constituents of the drug.[8]

CONCLUSION

In perspective of above described methods we came to conclude that environmental conditions, temperature, cultivated and wild plants, collection, drying, and their storage conditions have been influencing a lot in their quality control and to stabilize the active ingredients.

Production of crude drugs is a need of this modern age in Unani System of Medicine of the basis of modern parameters to promote U.S.M.

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Unani Medicine and Stability Studies – A Short Communication

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ABSTRACT

Unani drug formulations are being use since the time of Hippocrates. Unani Hakeem mentioned the Stability periods of Unani formulations based on their keen observations and organoleptic parameters. Therefore, the acute observations of the Unani classical authorities need to be substantiated with empirical evidence using scientific methodology.

Key words: Unani drug formulations, Hakeem's, organoleptic parameters, scientific methodology.

INTRODUCTION

As we observe in our routine life that all objects get spoiled after a specific period. All the living beings go through a cycle of birth, growth, reproduction and death. All the non-living substances that are grown or manufactured go through a life span in which they influence and are influenced by their environment. Everything made by human hands from the sublime Parthenon to the trivial milkshake is subject to decay. There is no existence of such a substance in the world, which is imperishable. Either it is our house where we resides or house hold substances like vegetables, fruits, rice or wheat, which we use in our daily routine life. Man is dubbed as the "most eminent of created beings" also has a life span after which he gets perished.[1]

Pharmaceuticals are no exception to this general statement. If there is any functionally relevant quality attribute of a drug product that changes with time, evaluation of this change falls within the purview of the pharmaceutical scientists and regulatory authorities who quantify the stability and shelf life of drug product. The rate at which drug products degrade varies dramatically. Some radiopharmaceuticals must be use within a day or so. Other products may become, if properly stored and packaged, retain integrity for a decade or more, although in many jurisdictions the maximum shelf life that a regulatory agency will approve for a drug product is five years. This restriction is hardly an onerous one, since even for a product with a five-year shelf life it is probable that over 95% of the product will sold and used within thirty months of manufacture, providing all involved in the distribution process obey the first law of warehousing: FIFO-first in, first out.[2]

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HISTORY AND UNANI MEDICINE STABILITY STUDIES

History of Greek medicine started with Pericles (561-430 BC) but very much distinguish figure was Hippocrates (460-377 BC), a member of the family of Asclepius (*Asqualibuis*). Hippocrates called as the father of medicine gave the fundamentals of medicine.[3] After the Hippocrates; Galen another prominent physician gave the scientific views to these fundamentals. Galen *(Jalinoos)* (131-201AD) who was attached to the roman court and all time influential writers was the first who mentioned about the shelf life of *Sufoof* (powder). Ghulam Jelani has mentioned a citation of *Jalinoss* in his book *Kitabul Murakkabat* that all powder retains their potency not more than two months.[4]

During the stint of Galen (*Jalinoos*) in second and third century AD Greek medicine transcended to its apotheosis but after Paul of Aegina (615-690) who was the last of compiler of Greek medicine descended to a stage of inactivity and then traveled to *Arab* world where it was preserved and nurtured. The first channel through which Greek medicine reached to the *Arab* world was the school of Alexandria (*Askandria*) and secondly the school of *Jundishapur* of Persia. In this connection the first book that

originally written in Arabic language was *Firdausul Hikmat* by Abul Hasan Ali Bin Sahel Rabban Tabri in ninth century A.D., had mentioned about shelf life of *Tiryaqe Akbar*. According to him *Tiryaqe Akbar* has efficacy up to 30 years and even more.[5] Abubakr Mohammad bin Zakaria Razi (865-925 A.D),[6] known to the west as Rhazez, was the most celebrated and most savant Arabic medical writer. *Razi* had mentioned shelf life of several single drugs in his book *Al Havi Fit Tib*. After *Razi* another Arab physician Ali Ibn-e-Abbas Majoosi (930-994A.D.), mentioned extensively about the shelf life of single as well as compound *Unani* formulation in his compilation *Kitab-Al-Mulki* that is famous with the name *Kamil- Al-Sanah*. He mentioned the shelf life of *Aqraas Ashqueel, Aqraas Afaai, Tiryaaq Arba,* and *Tiryaq Shalisa*[7] from two months to two years and shelf life of *Majoon kibrit* from six month to three years. The great philosopher, thinker and prominent physician of *Arabic medicine* Shaikh-Arrais-abu Ali Bin Sina (375-428 A.H.), has illustrated the stages of potency and degradation of *Tiryaq-e-Farooque*.[8]

Other profound *Unani* physicians like Sharfuddin Ismail Jurjani (531 AH)[4], Mohammad Ayyub Israeli[9], Mohammad Hadi Khan Mohammad Husain, Hakeem Akbar Arzani (1722 D)[5], and Kabiruddin (1894-1976 AD)[6] noted down the shelf life of certain Unani compound formulations. The observations related to Shelf life of drugs are seen originally in the text of Mohammad Hadi Khan Mohammad Husain.[10] The other writers have just followed the trends of their predecessors like *Hkm* Kabeeruddin and Azam Khan. Recently, it has become mandatory to evaluate stability studies of drugs to ascertain their purity and efficacy over time. Obviously, loss of drug constituents is of major importance in the stability studies of many pharmaceutical products. Unfortunately, one sometimes gets the impression that some regard this as only adverse effect of drug product stability. This is, of course, not true and for some products, losses of active ingredients are not critically variables that determines shelf life. However, it is certainly true that for many products loss of potency is of major importance. In general, we regard any product that contains less than 90% of label claim of drug as being of unacceptable quality. Therefore, for many drugs products, determination of the time that elapses before the drug content no longer exceeds 90% (when the product is stored in conformance to label instructions) is an essential element in determining shelf life. The long term testing should cover a minimum of 12 months' duration on at least three primary batches at the time of submission and should continue for a period sufficient to cover the proposed re-test period. Additional data accumulated during the assessment period of the registration application should submit to the authorities if requested. Data from the accelerated storage condition and, if appropriate, from the intermediate storage condition can be used to evaluate the effect of short term excursions outside the label storage conditions (such as might occur during shipping). Long term, and accelerated stability studies and, where appropriate, intermediate storage conditions for drug substances should evaluated in detail. The general case applies if the drug substance is not specifically cover by a subsequent section. Alternative storage conditions can be use if justified. If long-term studies are conducted at 25°C ± 2°C/60% RH ± 5% RH and "significant change" occurs at any time during 6 months' testing at the accelerated storage condition, additional testing at the intermediate storage condition should be conducted and evaluated against significant change criteria. Testing at the intermediate storage condition should include all tests, unless otherwise justified.[11, 12, 13]

As shown in above discussion that in most of Unani classical literatures have mentioned the stability periods of formulations so we have to evaluate them based on modern stability parameters.

CONCLUSION

Unani physicians have used Unani formulations since centuries, by their keen observation and experience, these authorities have noted about the expiry dates of various dosage forms. Presently, stability studies are a must for registrations of a drug product and to ensure the quality of a finished product, thus assessing stability of a product in itself is a foremost necessity.

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A Comprehensive Review of a Magic Plant, *Hippophae rhamnoides*

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ABSTRACT

Reliance on herbal medicines in the management of diseases is still much experienced by a large share of the people especially rural population due to their easy accessibility and cost effectiveness. Because of greater than before attentiveness regarding significance of conventional medicine in health care, research on medicinal plants would be valuable. *Hippophae rhamnoides* has been used customary in Chinese and Russian medicine for some decades. Today, a range of health products are being manufactured from *Hippophae rhamnoides*. Applications include skin disorders such as eczema, psoriasis, lupus erythematosus and dermatosus, cardiovascular diseases, cancer, burns, digestive tract disorders, anti-inflammation and UV radiations protecting effects. This review aims, (I) to refresh the importance of *Hippophae rhamnoides* to the medicinal plant researchers and (ii) to presents new information such as anti-sebum secretion effects of *Hippophae rhamnoides*.

Key words: *Sea Buckthorn*, Topical applications, Magic plant.

INTRODUCTION

It is postulated that there is a plant for every need on every continent. Remarkably this statement is true for example for soap preparation *saponaria officinalis* (soapwort) is used in Europe, *Yucca glauca (yucca)* in USA, *Sapindus indica (soapnut)* in India, *Phytolacca dodecandra (endod)* in Africa and *Quillaja saponaria (soap bark)* in South America.[1] *Hippophae rhamnoides (Sea Buckthorn)* is a deciduous, dioecious plant with numerous greenish-yellow flowers and bright orange, globular, ellipsoid fruit.[2] It is native to Eurupe, India, Nepal, Bhutan, Pakistan and Afghanistan. *Hippophae rhamnoides* shrub is 2 meter tall with 2-6 cm long leaves. It can be cultivated in deep, well-drained soil with pH 5.5-8.3. *Hippophae rhamnoides* plant can endure a temperature of extreme minimum of 40-43ºC.[3] The whole plant of *Hippophae rhamnoides* is important however berries are the most important part from which the juice is extracted. The berries (fruit) of *Hippophae rhamnoides* are maturated in three phases. The 1st phase is the accelerating seed growth, 2nd phase is the declining transition seed growth and the final 3rd phase is the known as berry maturation phase. In most part of the world including Pakistan the berries are ripened at the start of September. If the branches are not disturbed then the berries remain whole of the winter attached to the branches.[4] *Hippophae rhamnoides* plant has reputed medicinal uses include treatment of skin disorder, peptic ulcers, heart problems and tumors. Because of these characters, the products of *Hippophae rhamnoides* got attraction for medicinal and cosmetics use.[5] *Hippophae rhamnoides* plant has resistant to drought, cold, and alkali and salt. The complex root system with nitrogen fixing nodules of the *Hippophae rhamnoides* makes it an optimal pioneer plant in soil and water conservation area.[6]

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TAXONOMICAL CLASSIFICATION

It is proposed that taxonomical classification has made greater efforts to conserve existing plant names, for the benefit of phytochemists and other users. A brief taxonomical classification of *Hippophae rhamnoides* is presented in **Table 1.**

Some physical properties of *Hippophae rhamnoides*

The comparative physical properties of pulp oil and seed oil have been presented in **Table 2.**

Table 1: Taxonomical classification of Hippophae rhamnoides*

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	*Rosidae*
Order	*Rhamnales*
Family	*Elaeagnaceae*
Genus	*Hippophae*
Species	rhamnoides

Table 2: Some physical properties of *Hippophae rhamnoides*

S.No.	Property	Fruit oil	Seed oil
01	Acid value	8.8	10.0
02	Optical rotation	2.10	2.14
03	Refractive index	1.46	1.41

Plant and Fruit Morphology

Hippophae rhamnoides has been used for centuries in Eurasia as food (tea, beverages, jam etc.) as well as for ethnomedical remedies. The fruits are known to be a rich source of vitamins, carotenoids, flavonoids, and phytosterols. Plant fruit morphology of *Hippophae rhamnoides* is described in **Table 3**.

Chemical Constituents

Chemical composition of the *Hippophae rhamnoides* varies according to the origin, climate and method of extraction.[4] *Hippophae rhamnoides* consists of Fruit acids, ascorbic acid, Flavonoids, carotinoids, fatty acids and sugar alcohols.[2] Flavonoids are present in all parts of *Hippophae rhamnoides*. Fresh fruits contain 854 mg/100gm while dried leaves contain 3888 mg/100 gm of Flavonoids.The main Flavonoids in *Hippophae rhamnoides* are isorhamnetein, Quercetin, myricetin and kaempferol.[7]

Polyphenoles include flavonols, catechins, proanthocyanidins and chlorogenic acids.[8] The vitamin C concentration in *Hippophae rhamnoides* ranges from 28-2500 mg/100 gm of berries in various subspecies of *Hippophae rhamnoides*. The various factors, which affect the concentration of vitamin C, include temperature, harvesting time, origin and method of processing. Subspecies of *Hippophae rhamnoides* also contain vitamin A, vitamin B1, B2, vitamin K and vitamin P.[4] Seeds and berries have sufficient amount of tocopherols (vitamin E). The concentration of tocopherols and tocotrienols ranges from 100-300 mg/1000 gm in seeds and 110-150 mg/1000 gm of berries Yellow –orange color of the berries is due the presence of carotinoids. Carotinoids in seeds present in a concentration of 1/20-1/5 to that of berries.[6] Organic acids like malic acid and quinic acids are also present in the *Hippophae rhamnoides* juice. Minerals in *Hippophae rhamnoides* juice include potassium, the most abundant, Cu, Cd, Fe, Zn, Mg etc.[4] Fatty acids distribution in the mesocarp and seeds lipids is different. The main fatty acids are palmitoleic acid, palmitic acid, linoleic acid and oleic acid.[9]

Table 3: Plant and Fruit Morphology

Active Growth Period	Spring and Summer
Bloat	None
Coppice Potential	Yes
Fall Conspicuous	Yes
Fire Resistant	No
Flower Color	Yellow
Flower Conspicuous	Yes
Foliage Color	White-Gray
Foliage Texture	Coarse
Fruit/Seed Color	Orange
Fruit/Seed Conspicuous	Yes
Growth Rate	Rapid
Height, Mature	18.0 feet
Nitrogen Fixation	Medium

Extraction of *Hippophae rhamnoides*

Due the presence of polar flavonoids, it is usually extracted by water and methanol mixture a ratio of 1:1.[7] Dried branches, seeds and berries of *Hippophae rhamnoides* may also be extracted with 70% ethanol at room temperature.[10] For volatile constituents, the dried fruits of *Hippophae rhamnoides* can be obtained by subjecting it to steam distillation for 4 hours. The volatile constituents are then separated by chloroform and anhydrous sodium sulphate.[9] Choloform: Methanol mixture in a ratio of 2:1 is used for lipid extraction.[11] Fatty acids and other polar components may also be extracted using n-hexane.[12] Seeds of the *Hippophae rhamnoides* have been successively extracted using chloform, acetone and methanol in a soxhlet apparatus, however is should be noted that this type of extraction yields less amount of phenolic compounds as compared to methanolic extracts.[13]

Antioxidant Activity of *Hippophae rhamnoides*

In-vitro study of hydrophilic extract of *Hippophae rhamnoides* showed good antioxidant activity, which was similar to the antioxidant activity of the methanolic extract of *Hippophae rhamnoides*[14]. Ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids and polypenols in the seeds, leaves and berries of *Hippophae rhamnoides* have been detected as natural antioxidants.[15] The antioxidant activity of *Hippophae rhamnoides* may be due to its higher phenolic and flavonoid contents.[13] *Hippophae rhamnoides* provides an excellent source of essential fatty acids that is 70% of its compsition. The seed oil is used as anti ageing, anti inflammatory, antioxidants and as natural UV blocking agent in cosmetic formulations.[16] The inhibitory effects of alcoholic leaf and fruit extract of *Hippophae rhamnoides* have been investigated to inhibit

the oxidative damage induced by chromium. Alcoholic leaf and fruit extract were found to prevent chromium induced free radical production and restored the antioxidant status. It has been shown that fruit of *Hippophae rhamnoides* inhibit nicotine induced oxidative stress. It has been demonstrated by various in-viv, in-vitro studies that *Hippophae rhamnoides* possesses antioxidant activity.[17]

Uses of *Hippophae rhamnoides*

Uses in traditional medicine

Hippophae rhamnoides juice is an important source of some valuable chemicals such as vitamin C, tocopherolmcrotrients, organic acids and polyunsaturated fatty acids. The juice of *Hippopha e rhamnoides* was the common medicine used in ancient.[4] The leaves and fruits have been used as antiseptic and wound healing as well as in the treatment of ulcers in folk medicines in Turkey.[9] T.Beveridge et al reported that *Hippophae rhamnoides* has valuable medicinal importance such as it is used in the treatment of skin disorder resulting from bed incarceration, peptic ulcers (both stomach and duodenal ulcers) and cardiovascular disorders.[5] For the past 5 decades *Hippophae rhamnoides* has been used for the treatment of radiation damage, inflammation and burns in Chinese folk medicines.[13] *Hippophae rhamnoides* oils have been used in natruceuticals, natural medicines and cosmetics as raw materials in Russia for some decades.[6] *Hippophae rhamnoides* oil extracts have also been used in the treatment of skin disorders such as eczema, psoriasis, lupus erythematosus and dermatosus.[17] *Hippophae rhamnoides* has been used in various part of the world as traditional medicine for the treatment of indigestion, cough and blood sepsis.[10]

Pharmacological effects

Guliyew et al have briefly reviewed pharmacological effects of *Hippophae rhamnoides* plant.

Antimicrobial and antitumoral effects

The phenolic compounds of *Hippophae rhamnoides* have the inhibitory effects against Gram-negative bacteria.[17] 70% *Hippophae rhamnoides* branches extract has proven activity against TPA induced tumor. This activity is because of three phenolic compounds such as catechin, gallocatechin and epigallocatechin.[10]

Antiulcerogenic effect

Hexane extract of *Hippophae rhamnoides* has activity against indometacin; stress and ethanol induced gastric ulcer.[17]

Liver diseases

According to Zhao et al *Hippophae rhamnoides* could be used to protect liver from damage by calcium tetrachloride. Combining *Hippophae rhamnoides* juice with antivirus can shorten the normalization time of serum ALT.[18]

Dermatological effects

Hippophae rhamnoides has beneficial effects against dermatological disorders such atopic dermatitis.[17]

Anti-sebum secretion effects

Naveed et al has reported the anti-sebum secretion effects of *Hippophae rhamnoides* by formulating a topical skin-care cream. They demonstrated that type 1-α reductase converts testosterone into more potent dihydrotestosterone, which results in the enlargement of sebaceous gland, leading to secreting high level of sebum. The polyphenol plant extract regulates the extreme sebum secretion. Topically applied oleic and linoleic acids have proved to inhibit type 1-α reductase. Polyphenoles in *Hippophae rhamnoides* include flavonols, catechins, proanthocyanidins, and chlorogenic acids, whereas the main fatty acids of *Hippophae rhamnoides* are palmitoleic acid, palmitic acid, linoleic acid, and oleic acid which regulate sebum secretion by inhibiting type 1-α reductase.[19]

Miscellaneous effects

Recent studies have focused on the healthy functions of aromatic and medicinal plants, which have antioxidant, antimicrobial, and mutagen properties.[20] Pathogenesis of alcoholic liver disease is mainly due to the generation of an excessive amount of reactive oxygen species (ROS). Antioxidants of plant origin have been reported to either inhibit or prevent the development of fundamental cellular disturbances.[21] V.B Guliyew has reported that *Hippophae rhamnoides* has effects on platelet aggregation, effects on blood lipids, electrophysiological effects as well as radioprotective effects have also been shown.[17]

CONCLUSIONS

Research on medicinal plants is increasing day by day. *Hippophae rhamnoides* is a magic plant as it contains a biodiversity of both nutritional as well as medicinal constituents. Targeted based studies with concentration on mechanism of action, lethal dose/effective dose and bioavailability mechanisms need to be conducted in future to explore scientifically the hidden potential of this magic plant so that the ill community gets maximum benefits from our traditional system of medicine. It is hoped, this review will encourage more attention towards research and more conviction towards utilization of herbal medicines.

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