Inhibition on Urease and Thermal Induced Protein Denaturation of commonly used Antiulcer Herbal Products. Study based on *in-vitro* assays

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ABSTRACT

Background: *In-vitro* urease inhibitory and thermal induced protein denaturation inhibitory activities was performed for two commonly used herbal products Endemali and Akseer ULCER in the treatment of ulcers. **Objectives:** To evaluate the antiulcer potential of two commonly used herbal products, Endemali, Akseer ULCER. **Material and Method:** In urease inhibitory assay, enzyme solution, extract, diferent regaents added and absorbance was measured at 630 nm (50 min, pH 8.2) and thiourea used as standard. In protein denaturation assay, the egg albumin was mixed with different concentration of test compounds, buffer absorbance was measured. Aspirin was used as standard. **Results:** The Endemali had a profound effect on the urease activity in a concentration dependent manner with EC_{50}value of 0.468 mg/ml. The Akseer ULCER antagonized the urease activity markedly with EC_{50}value of 0.374 mg/ml.These tested herbal products caused marked inhibition of thermal induced protein denaturation in a concentration dependent manner. The potency in the form of $EC_{50}$for Endemali, Akseer ULCER was measured as 323, 337 μg/ml respectively. **Conclusion:** In short, the tested herbal drug showed strong inhibition on urease activity and inhibition on thermal induced protein denaturation thus our study validated their uses in the treatment of ulcers.

Key words: Akseer ULCER, Antiurease activity, Endemali, *in-vitro* assay, Thermal induced protein denaturation.

INTRODUCTION

The traditional Unani System of Treatment is the integral part of Pakistani community.[1-2] The physician in Unani system of treatment is called Hakim/Tabib and the system as Hikmat/Tibb. The federal government through Unani, Ayurvedic and homeopathic (UAH) Practitioners Act, 1965, regulates the Unani System of Medicine.[3-4] National Council of Tibb and National council for Homeopathy are established as corporate bodies under section 3 of the said Act to promote and popularize the traditional system of education. The registered Hakims and vaids throughout the country are estimated at 39, 584 and 455 respectively. These healthcare professionals are produced by 125 recognized homeopathic medical colleges in the country.[5-7] However, reasonable number of unregistered Hakims are too involved in the practice; based on the empiric knowledge came from ancestors. Like other countries of the world, the ulcer is very common in Pakistani community and badly affecting the lifestyle of people. Along with allopathic drugs, Unani medicines are benefiting large population of the country. However, mostly these formulations are used without a scientific background. Therefore, the current was designed to evaluate the antiulcer potential of two commonly used herbal products, Endemali, Akseer ULCER. The antiulcer potential was rated in terms of urease inhibitory effects of

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DOI: *10.5530/pj.2015.3.1*

these drugs in an *in-vitro* assay.

MATERIALS AND METHODS

Drugs Material

The Endemali strips of Hamdard Laboratories (WAQF) Pakistan, Akseer ULCER tablet of Dawakhana Hakim Ajmal Khan (Pvt) Ltd was purchased from local market.

Chemicals

Urea (Sigma-Aldrich), Sodium Nitroprusside, Phenol Red(BDH Chemicals Ltd, England), Thiourea, Sodium Dihydrogen Phosphate, (Merck, Germany), UreaseJack beans (Avonchem Ltd, UK), Sodium Hypochlorite (HC Haq Chemicals, Pakistan), Dimethyl Sulfoxide(UNI-Chem).

Urease inhibitory assay

Exact 25 ml of enzyme (Jack Bean Urease) solution and test compounds (5 ml, 0.5 mM concentration) were incubated for 15 min at 30°C (Tariq *et al*., 2011). The aliquotwas taken after 15 min and again incubated with 55 µL of buffers containing urea (100 mM) for 15 min at 30 °C. Ammonia production was measured as an urease activity by indophenol method as described earlier (Khan *et al*., 2013c). Final volumes were maintained as 200 ml by adding 45 ml phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprussside), and of alkali reagent (70 ml, 0.5% w/v NaOH and 0.1% active chloride NaOCl). The increase in absorbance was measured at 630 nm after 50 min at pH 8.2. The results (change in absorbance per min) were calculated spectrometrically on different concentrations of drugs. Thiourea was used as the standard inhibitor and percentage inhibitions were calculated as follows

$$\% \text{ Inhibition} = 100 - (OD_{testwell}/OD_{control}) \times 100.$$

The IC_{50} values were calculated using statistical software, Graph Pad version 6.

In-vitro protein denaturation assay

The protein denaturation assay was used for the estimation of antiinflammtory potential of herbal product, Endemali, Akseer ULCER. The egg albumin (0.2 ml) was mixed with varying concentrations of decoctions (2 ml, 50-500 µg/ml) and phosphate buffered saline (2.8 ml, pH 6.4) to get a reaction mixture of 5 ml. The mixtures were incubated at 37ºC ± 2 for 15 min and then heated at 70ºC for 5 min. The resulting solutions were cooled and absorbance was taken on a spectrophotometer at 660 nm by using vehicle as blank (Chandra *et al*., 2012, Chatterjee *et al*., 2012, Khan *et al*., 2014). The distilled water served as control while aspirin as a standard. Finally, the % inhibition of protein denaturation was calculated.

$$\text{Protein denaturation (\%)} = \frac{\text{Control Absorbance} - \text{Test Absorbance}}{\text{Control Absorbance}}$$

The IC_{50} values were calculated using statistical software, GraphPad PRISM 6.

Statistical analysis

The resulting data were expressed as the mean ± SEM (n=3) in each group. To determine the differences between groups, one-way analysis of variance(ANOVA) was performed (Graph Pad version 6) usingtheleast significant difference (LSD) test at P<0.5. EC_{50} values were calculated with the help of curve-fitting program.

RESULTS

Effect of tested herbal products in urease inhibitory assay

The results of tested herbal products in urease inhibitory assayare displayed in Figure 1. Endemali had a profound effect in a concentration dependent manner on the urease activity (Figure 1a). The maximum attenuation (78%) was observed at 3 mg/ml. The EC_{50} was calculated as 0.468 mg/ml (table 1). The results of Akseer ULCER against Jack Bean urease is illustrated in (Figure 1b). It antagonized the urease activity which was dependent on drug concentration. In terms of percentage, maximum inhibition was 87% at 3 mg/ml. The calculated EC_{50} was 0.374 mg/ml (table 1). As shown in (Figure 1c), the powder drug also showed concentration dependent antagonism of the urease. The maximum inhibition was 59% inhibition of urease at 3 mg/ml. The calculated EC_{50} was 1.80 mg/ml (table 1). However, Thiourea showed most potent effect (Figure 1d) with EC_{50} value of 0.026 mg/ml (table 1).

Effect of tested herbal products in thermal induced protein denaturation assay

The effect of tested herbal products in thermal induced protein denaturation assay at various concentrations is illustrated in Figure 2. The endemali caused concentration dependent inhibition of heat-induced protein denaturation

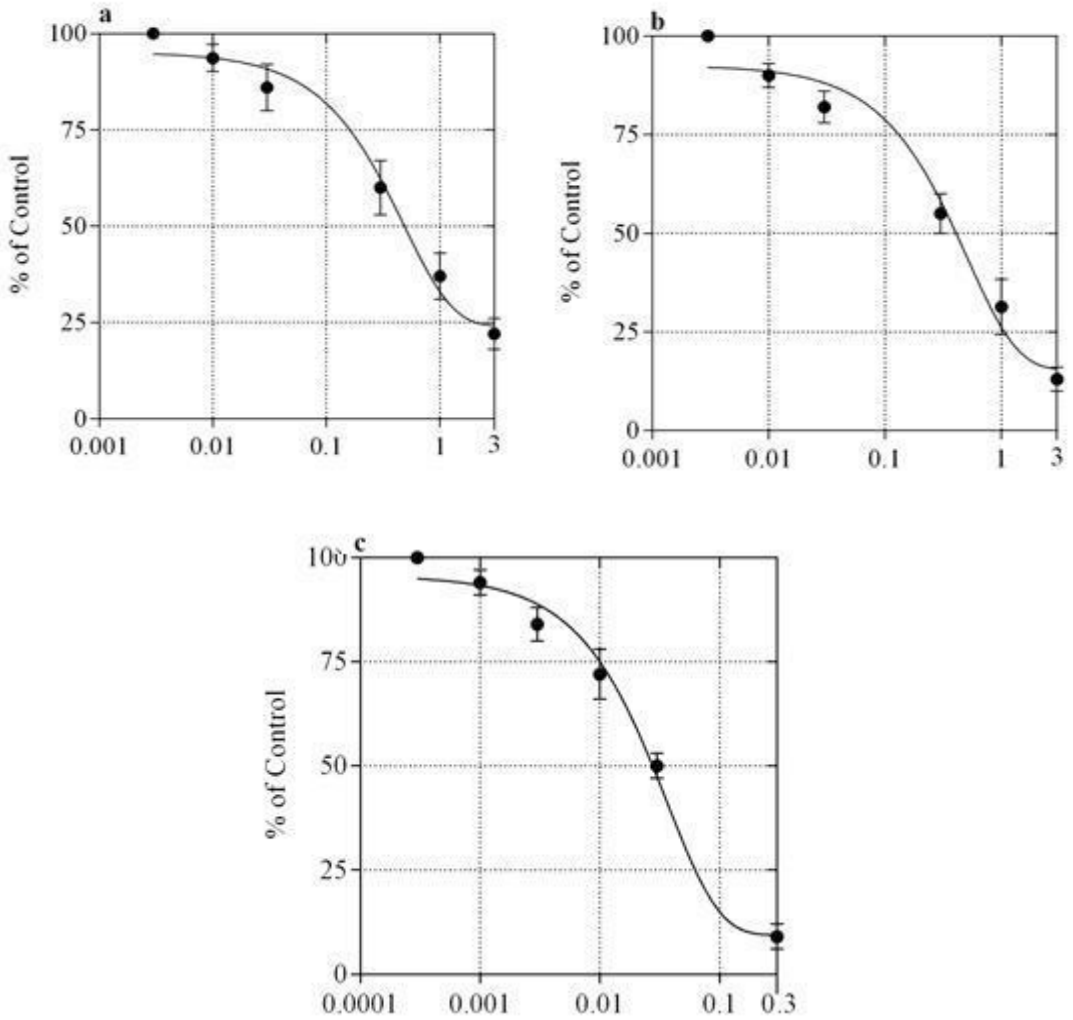

Figure 1: The percent effect of herbal products on urease inhibition. (a) Endemali, (b) Akseer ULCER, and (c) Thiourea. The resulting data were expressed as the mean ± SEM (n=3) in each group.

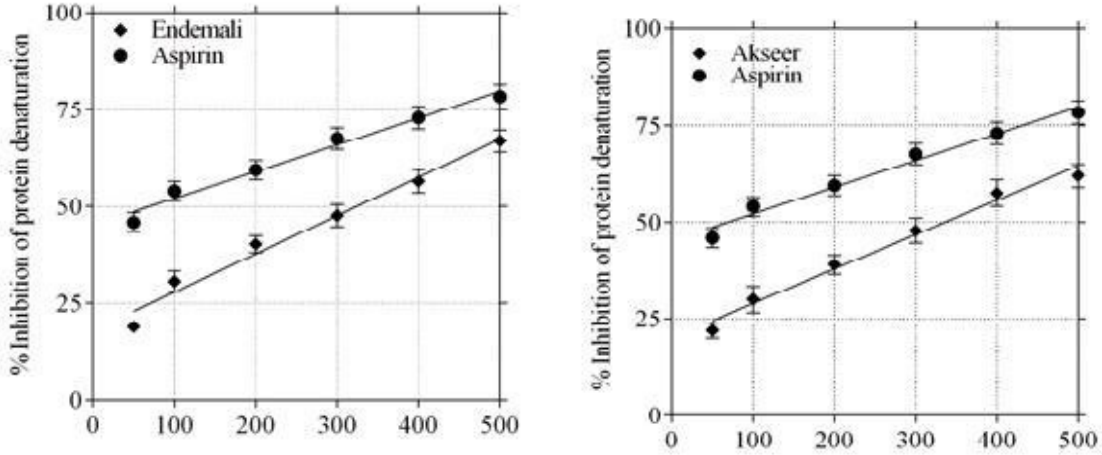

Figure 2: The percent inhibition of effect of herbal products on thermal induced protein denaturation assay. The resulting data were expressed as the mean ± SEM (n=3) in each group.

Table 1: Half maximual concentration (IC_{50}:mg/ml) of herbal drug in urease inhibitory assay. The resulting data were expressed as the mean ± SEM (n=3) in each group.

Drugs	EC_{50} (mg ml)
Endemali	0.468
Akseer ULCER	0.374
Thiourea	0.026

Table 2. Half maximual concentration (IC_{50}:mg/ml) of herbal drug in thermal induced protein denaturation assay. The resulting data were expressed as the mean ± SEM (n=3) in each group.

Drugs	EC_{50} (µg ml)
Endemali	323
Akseer ULCER	337
Aspirin	65

with maximum attuneation of 67% at 100 µg/ml and EC_{50} of 323 µg/ml (table 2). The akseer ULCER also exhibited marked inhibition on thermal induced protein denaturation with maximum effect of 62% at 100 µg/ml and EC_{50} of 337 µg/ml (table 2). The various concentrations of powder drug showed profound inhibition on thermal induced protein denaturation with maximum effect of 68.50% at 100 µg/ml and EC_{50} of 297 µg/ml.

DISCUSSION

The results of our study revealed profound effect on commonly used herbal formulations on the expression of urease in an *in-vitro* assay.

Urease (urea amidohydrolase) is usually found in different bacteria, fungi, algae and plants, an enzyme that catalyzes the hydrolysis of urea to ammonia and carbamate, which is the final step of nitrogen metabolism in living organisms.[8] Carbamate rapidly and spontaneously decomposes, yielding second molecule of ammonia. These reactions may causes significant increase in pH and are responsible for negative effect so urease activity in human health and agriculture.[9-10] From the medical view point, infections induced by these bacteria such as *Helicobacter pylori* and *Proteus mirabilis* usually have a high urease activity. Urease is vital to *H. pylori* metabolism and virulence, as necessary for its colonization of the gastric mucosa, and is a potent immunogen that elicits a vigorous immune response. This enzyme is used for taxonomic identification and for diagnosis and follow-up after treatment, and is a vaccine candidate. Ureas represent an interesting model for metalloenzyme studies. *H. pylori* contributes in urinary tract and gastrointestinal infections, probably augmenting the severity several pathological conditions like peptic ulcers and stomach cancer. Ureases are also involved in the development of urolithiasis, pyelonephritis, hepaticencephalopathy, hepatic coma and urinary catheter encrustation.[11-12]

In this regards, targeting urease for treating pathogenic disorders caused by it may open a new line of treatment for infections caused by urease producing bacteria. The results of our study showed profound inhibition of urease (Jack Bean) by two commonly used herbal products in a concentration dependent manner. Therefore, our study provided the scientific basis for the traditional uses of these herbal agents of plant origin as antiulcer.

The development of an inflammatory response is a complex but well regulated process. Arachidonic acid is a polyunsaturated fatty acid that liberated from cell membrane phospholipids via the hydrolysis by phospholipase A2 enzymes (PLA2). The arachidonic acid is then metabolized by two distinct enzymatic pathways; cyclooxygenase (COX) in to prostaglandins (PGs) and lipoxygenase in to leukotrienes.[13-15] Prostaglandins (PGs) are members of the eicosanoid family produced by almost all cells of the human body; the principal mediator of inflammation in most of the inflammatory diseases.[16-17] The inflammatory mediators approach from plasma proteins or cells including mast cells, platelets, neutrophils and monocytes/macrophages which are activated by bacterial products or host proteins. They bind to specific receptors and elicited vascular permeability, neutrophils chemotaxis, stimulate smooth muscle contraction, excite pain or mediate oxidative damage. Most of the mediators are short - lived but produce harmful effects.[18-19] The non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed medicine in the management of inflammatory conditions. Clinically, they are useful for the symptomatic relief[20-21] by acting through several mechanisms though causing various side effects.[22-23]

It was already proved that conventional NSAID's like phenyl butazone and indomethazine do not act only by the inhibition of endogenous prostaglandins production by blocking cyclo oxygen as enzyme but also by prevention of denaturation of proteins.[24-25] The protein denaturation is a practice in which proteins are unable to maintain their structural integrity in the presence of external stimuli such as strong acid or base, concentrated inorganic salt, an organic solvent or thermal treatment. It has been observed that proteins lose their biological potency upon denaturation. Denatured proteins are considered

as one of the inflammatory mediator therefore, agents that cause prevention of precipitation of denatured protein aggregates and protein condensation are useful in diseases like rheumatic disorders, cataract and Alzheimers disease.[26] In the current study, the tested herbal products demonistrated marked attenuation of urease activity at various test concentrations. Additionally, the urease inhibition of these herbal products was augmented by their strong inhibition on thermal induced protein denaturation. Thus urease inhibition coupled with anti inflammtory effect could be more effective the rapeutic approach in the treatment of ulcers as inflammation is primerly observed with hyperacidic conditions.

CONCLUSION

It is concluded that the tested two herbal Pakistani drugs showed marked inhibition against urease. The study therefore, provided scientific evidence for their use in the treatment of ulcers. Furthermore, the inhibition on thermal induced protein denaturation could be more effective therapeutic approach in the treatment of ulcers.

CONFLICT OF INTEREST

The author has no conflict of interest.

ACKNOWLEDGEMENTS

We are thankful to the Department of Pharmcy, AIMST University, Malyasia technical support.

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Pogostemon cablin (Blanco) Benth. (*Lamiaceae*): It's Ethnobotany & *in vitro* regeneration

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ABSTRACT

Objectives: Since the beginning of human civilization various herbal medicines are employed for healing human and animal. *Pogostemon cablin* (Blanco) Benth., locally known as *Patchouli* (Assamese) is a very important medicinal plants belongs to mint family i.e. *Lamiaceae*. The main aim of this study was to collect ethnobotanical information's and to study its *in vitro* regeneration results. All possible ethnobotanical literatures have been cited here. **Methods:** *In vitro* propagation was achieved from leaf and nodal explants of *Pogostemon cablin* on MS medium. **Results:** Callus development and *in vitro* axillary shoot formation was successfully made in MS basal medium containing BA (4.0 mg/L), NAA (2.0 mg/L) + IAA (1.0 mg/L) and BA (3 mg/L) + IAA (1 mg/L). MS basal medium containing IBA (0.1/L) and Kn (2.5 mg/L) was best for induction of multiple shoots within 4 weeks of culture. Combination of NAA (0.1 mg/L). Kn (0.1 mg/L) and CH (100 mg/L) was best for callus induction which later on formed multiple shoots and caused elongation of roots. Micro shoots of varied length were produced on MS medium. Rooted plantlets were successfully acclimatized in green house for 1 month and then were transferred to the field. **Conclusion:** It can be concluded that *pogostemon cablin* has immense ethno botanical importance. For its rapid multiplication, *in vitro* technique was found very successful. In MS medium supplemented with Kn 2.5 mg/L and IBA 0.1mg/L found maximum multiplication rate. In this proportion rates of shoot generation, leaf, rooting, callus formation was maximum.

Key words: Ethnobotany, *in vitro* study, MS medium, *Pogostemon cablin.*

Highlights of the paper :

- *Pogostemon cablin* (Blanco) Benth., locally known as Patchouli (Assamese)is a very important medicinal plants belongs to mint family i.e. Lamiaceae.
- A plant of immense ethno-botanical importance.
- After several trials, it was found that MS medium with Kn 2.5mg/L and IBA 0.1 mg/L gave a maximum multiplication rateof shoot, root and callus initiation. Hence, recommended.

INTRODUCTION

Medicinal plants have provided modern medicine with numerous plant derived therapeutic agents.[1] In aromatherapy, lots of medicinal plants are used, in which volatile plant materials are used, known as essential oils, and other aromatic compounds for the purpose of altering a person's mind, mood, cognitive function or health.[2] The anti-microbial effects has demonstrated from tea tree, but there is still a lack of clinical evidenced monstrating efficacy against bacterial, fungal, or viral infections.[3] Human culture has been augmented by plants and plant products since time immemorial. Perhaps ethnobiology is the first science that originated with the evolution of men in this planet.[4] The plant patchouli belongs to the family *Lamiaceae* under the genus *Pogostemon*, which comprises about 130 species and native to tropical Asia and is widely grown in India, Malaysia, Philippines, Indonesia and Singapore.[5-7]

The patchouli oil, obtained by steam distillation of shade-dried leaves is commercially used in perfumes and cosmetics.[8,9] Patchouli has insecticidal activities, anti-fungal and bacteriostatic properties.[10-12] Patchouli plants regeneration from stem tip, leaf and nodal callus, protoplasts

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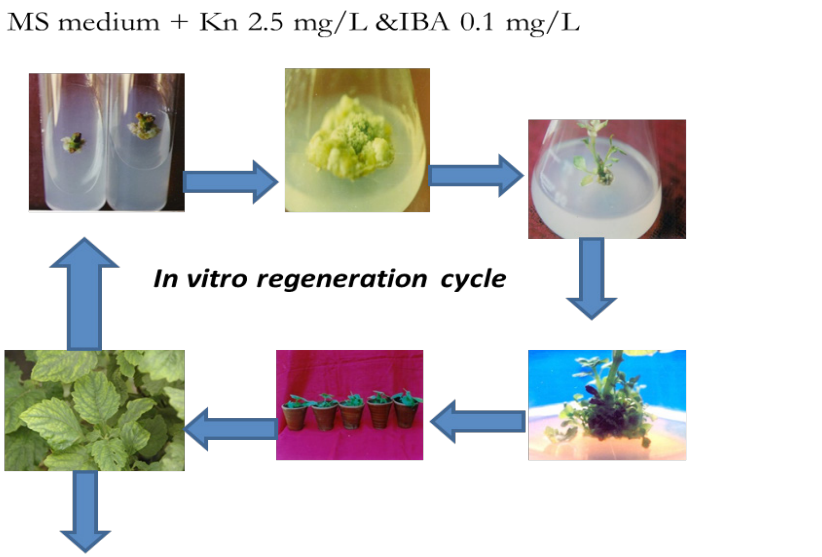
DOI: *10.5530/pj.2015.3.2*



Graphical Abstract

encapsulated in alginate beads, somatic embryogenesis have been reported earlier.[13-17]

CLASSIFICATION[18,19]

- Domain : Eukaryota
- Kingdom : Plantae-Plants
- Subkingdom: Tracheobionta- Vascular plants
- Super division: Spermatophyta – Seed plants
- Division: Magnoliophyta – Flowering plants
- Class: Magnoliopsida – Dicotyledons
- Subclass: Asteridae
- Order: Lamiales
- Family: Lamiaceae– Mint family
- Genus: Pogostemon Desf. – pogostemon
- Species: Pogostemon cablin (Blanco)Benth. – Patchouli (Common name)

VERNECULAR NAMES[20-22]

Malaysia: Dhalum Wangi, Tilam Wangi, Nilam

English: Patchouli

Indonesia: Nilam Wangi (General), Nilam (Acheh), Singalon (Batak)

Thailand: Phimsen (Bangkok)

Vietnam: (Ho (aws) ch (uw) (ow) ng)

Philippines: Kabling (Tagalog); Katluen (Bisaya) Kadlum (Bikol, Bisaya, Sulu)

China: GuangHuo Xiang

Korea: Hyangdulkkaephul

India: Pachi (Sanskrit); Pachauli (Hindi); Pachapat,Patchouli (Bengali); Pachila, Kattam (Malayalam);Pacha, Sugandhipandi (Gujarati);Panch (Marathi)

French: Patchouli

Spanish: Pachuli

ETHNOBOTANICAL REPORTS

Cooling and tonic, is used in asthma cough and debilitating diseases.[23] Whole plant ash used to kill worms in animal wounds.[24] Because of its primary antiseptic properties, it is used to treat athlete's foot, dandruff, wounds and scars. It gives relief from constipation and acts as an antidote against insect bites temporarily. Patchouli alcohol is a fragrance ingredient used in decorative cosmetics, fine fragrances, shampoos, toilet soaps, non-cosmetic products such as household cleaners and detergents.[25] It also used as daily dosage along with other herbs for treatment of asthma.[26]

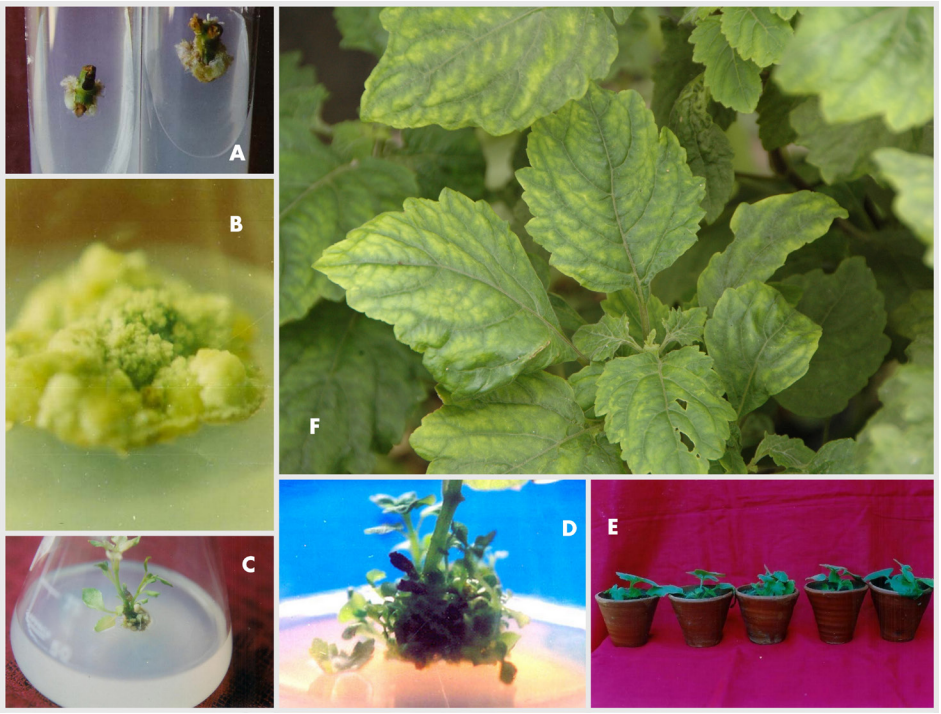


Figure 1: A. Callus initiation; B. Callus development; C. Root initiation from callus D. Multiple shoot formation; E. transferred to pots; F. A *Pogostemon cablin* twig.

MATERIALS AND METHODS

Plant Material

Nodal stem with axillary buds were used as explants were surface sterilized with 70% alcohol and then with 0.01% $HgCl_2$ solution. The explants were immersed in 100 ml of tap water containing 1-2 drops of extra for 5 minutes and later washed with sterile distilled water. The stem segments were further cut into 1 cm pieces having one node and were used as explants.

Culture media and condition[27]

Medium supplemented with PGRs–BA, 2, 4-D, IAA, IBA, Kn, and NAA was used under aseptic condition. Myo-inositol 100 mg/1 (wt/vol), sugar (3%), casein hydrolysate (100 mg/1) were used in the media for shoot bud proliferation. The p^H of the media was adjusted to 5.7 and solidified with agar (0.8%). The medium was sterilized in an autoclave at 121°C for 15 minutes. Aseptic condition was maintained throughout the whole operation. Cultures were maintained at 25 ± 2°C under fluorescent light of about 2500–3000 lux with 16 hr photoperiod/day.

For further multiplication

For acclimatization and transfer of plants to soil rooted shoots were removed from culture flasks and washed dipped in 0.2% bavistin fungicide for 5 minutes and plantlets were potted in a sterilized mixture of garden soil and sand. They were irrigated with half strength MS solution for 1 week and subsequently with sterile distilled water. The plantlets were acclimatized under laboratory condition before transferring to Green House and then to natural condition.

RESULTS AND DISCUSSION

In the present study, different combination of BA, IBA, IAA, Kn, NAA and 2, 4-D were tried. Among the combination tried, MS medium supplemented with Kn 2.5 mg/L and IBA 0.1 mg/L gave a maximum multiplication rate with 72.33 ± 0.80 shoot number (Figure 1, D), 15.64 ± 0.44 cm shoot length (Figure 1, C), 5.7 ± 3.10 leave no and 3.3 ± 1.28 no's of roots (Table 1).Callus formation was also maximum in this concentration (Figure 1, A & B). It was followed by Kn (0.5 mg/L)+IBA (0.5 mg/L) concentration which gave multiplication rate 30.0 ± 3.08 shoot number, shoot length 2.2 ± 0.57cm, 5.25 ± 2.70 leave no and 20.0 ± 0.58 no's of roots with well-developed callus.

When BA was added in MS medium at concentration ranging from 0.1 to 4.0 mg/L with NAA and IAA not much development of shoots observed on lower concentration. But in BA (4.0 mg/L) with NAA (2.0 mg/L) and IAA (1.0 mg/L) resulted higher rate of shoot multiplication with 58.0

Table 1: Effect of Plant Growth regulators on MS medium

MS Medium+PGRS (mg/L)							Intensity of Development			Observation after 30 days			
BA	NAA	IAA	IBA	Kn	2,4-D	CH	C	S	R	No.of shoots	Shoot length	No.Of leaf	No.of roots
0.1	05	-	-	-	-	-	++	++	-	2.3±0.57	2.53±0.68	2.5±0.70	1.5±0.57
0.5	1.0	-	-	-	-	-	++	++	-	1.5±0.57	2.63±0.89	5.68±1.49	0
1.0	0.5	-	-	-	-	-	++	+++	++	14.3±0.66	06.5±0.31	4.75±1.705	2.0±0
3.0	-	1.0	-	-	-	-	+++	+++	++	17.00±.44	05.2±0.17	1.60±0.06	18.0±0.58
4.0	2.0	1.0	-	-	-	-	+++	+++	+	58.00±1.0	09.17±0.37	1.37±0.03	25.3±0.88
-	0.1	-	-	0.1	-	50	+++	+++	+++	27.67±2.02	7.80±0.15	1.23±0.03	5.0±0.40
-	0.5	-	-	0.1	-	50	+	+++	++	16.00±01.0	6.13±0.20	1.17±0.06	4.6±1.53
-	1.0	-	-	0.1	-	50	+	++	+	12.33±0.33	6.03±0.13	1.27±0.03	3.3±0.57
-	2.0	-	-	0.1	-	50	+	+	+	10.00±0.58	9.97±0.23	1.47±.03	1.5±0.57
-	3.0	-	-	0.1	-	50	+	+	+	1.5±0.57	1.17±0	1.5±0.29	1.25±0.50
-	-	-	-	0.1	0.1	100	+	++	++	1.5±0.57	1.62±0.85	3.5±1.29	1.50.57
-	-	-	-	-	1.0	100	+	+++	-	1.75±0.50	1.62±0.63	5.0±0.82	-
-	-	-	-	1.0	0.5	100	+	++	++	1.25±0.50	1.17±0.69	3.5±1.29	2.00.82
-	-	-	-	1.0	1.5	100	+	+++	-	2.5±0.57	2.25±0.95	2.5±0.57	-
-	-	-	-	1.0	2.5	100	+	+++	-	1.5±0.57	1.12±0.57	1.5±0.57	-
-	-	-	0.5	0.1	-	-	+	+++	+	2.3±0.57	2.53±0.68	5.68±1.49	1.5±0.57
-	-	-	1	0.1	-	-	+	++	-	1.5±0.57	2.63±0.89	4.75±1.70	0
-	-	-	0.5	0.5	-	-	+	+++	+	30.0±3.08	2.2±0.57	5.25±2.70	20.0±0.58
-	-	-	0.5	1.5	-	-	+	+++	-	2.0±0.0	2.65±0.96	4.42±1.65	0
-	-	-	0.1	2.5	-	-	+	+++	+	72.33±0.88	15.64±0.44	5.7±3.10	3.3±1.28

C- Callus, S- Shoot, R- Root ; +++ →Excellent, ++ → Good, + → Positive

± 1.0 shoot number, 0.9 ± 0.37 cm shoot length, 1.37 ± 0.03 leaf number and 25.3 ± 0.88 nos. of root. Also callus formation was excellent in this combination. Even lowering the kinetin concentration to 0.1 mg/L with NAA 0.1 mg/L and CH 50 mg/L a good number of shoots 27.67 ± 2.02, 7.80 ± 0.15 cm shoot length, 1.23 ± 0.03 leave no and 5.0 ± 0.40 no of roots were noticed at laboratory stage (Table 1).

The callus induction was found to be good with combination of Kn (1.0 mg/L)+2,4-D (2.5 mg/L)+CH (100 mg/L). The combined effect of NAA (0.1 mg/L)+Kn (0.1 mg/L)+CH (100 mg/L) produced best callus which later developed both shoot and root in MS medium. With increase in concentration of auxin the two axillary buds developed only a few shoots. Development of callus was also found in combination of BA (3.0 mg/L) + IAA (1.0 mg/L), Kn (1.0 mg/L)+2, 4-D (1.5 mg/L, 2.5 mg/L)+CH (100 mg/L) in MS medium. Multiple shoot formation also resulted in combinations of BA (3.0 mg/L)+IAA (1.0 mg/L) and in BA (1.0 mg/l)+NAA (0.5 mg/L) After 40 days plants are transferred to pots for better growth (Figure 1, E & F).

CONCLUSION

From this study it can be concluded that *pogostemon cablin* has immense ethno botanical importance. Due to its enormous importance and demand, the mass propagation through *in vitro* technique was found very successful. After successful experiments with lots of combinations, it has been found that MS medium supplemented with Kn 2.5 mg/L and IBA 0.1 mg/L gave a maximum multiplication rate. In this proportion rates of shoot generation, leaf, rooting, callus formation was maximum.

ABBREBIATION

MS : Murashige and Skoog (1962) medium,
BA : N^6 Benzyladenine,
IBA : Indole-3-Butyric Acid,
IAA : Indole-3-Acetic Acid,
NAA : Naphthelene Acetic Acid,
Kn : Kinetin,
2,4-D : 2,4 Dichloro Acetic Acid,
CH : Casein Hydrolysate

CONFLICTS OF INTEREST

The authors are declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors are thankful to the Tezpur University and Department of Botany Gauhati University for providing necessary facilities for conducting the experiments.

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Effectiveness of Sidr Honey on the prevention of ethanol-induced gatroulcerogenesis: role of antioxidant and antiapoptotic mechanism

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ABSTRACT

Background: Sider (*Ziziphus spina-christi* (L.) Desf.) Honey has been used for the treatment of gastrointestinal disorders including peptic ulcer. **Aim of the study:** The mechanism of the antiulcer effect of sider honey was studied placing emphasis on its role to block oxidative damage and apoptosis during ethanol-induced gastric ulceration in rats. The mechanism of the antiulcer effect of sider honey was studied placing emphasis on its role to block oxidative damage and apoptosis during ethanol-induced gastric ulceration in rats. **Materials and methods:** Experimental animals were orally treated with sidr honey (100, 250 and 500 mg/kg, respectively) or omeprazole and subsequently exposed to 95%ethanol (5 mL/Kg, orally) to induce acute gastroulcerogenesis. Effectiveness of sidr honey was evaluated using ulcer index, pH of gastric juice, mucus content, morphological analyses, glutathione assay and malondialdehyde level. The anti-apoptotic role of sidr honey was studied using immunohistochemical staining of gastric tissues using monoclonal antibodies of Bax pathway. **Results:** Dose-response studies in ethanol-induced ulcer indicate that sidr honey significantly blocks gastric lesions at lower dose (100 mg/kg). Lipid peroxidation and glutathione depletion were significantly inhibited by sidr honey. Sidr honey modulated the immuno-expression of mitochondrial associated protein (Bax). **Conclusion:** Thus, sider honey plays a considerable role in gastro protection by acting as a potent antioxidant and antiapoptotic agent. Future study is required to explore its potential clinical usage.

Key words: Antiapoptotic Mechanism, Antioxidant Agents, Gastro protection, Monofloral Nectar, Sidr Honey.

INTRODUCTION

The ethno-medicinal and ethno-nutritional uses of honey have been historically known.[1,2] Modern biomedical research has demonstrated that honey is a considerable natural antioxidant and has potential remedial value in the treatment of tumors, diseases of heart and eye, and inflammation.[3] The curative potential of honey involves free-radical scavenging activities and antibacterial properties, as well as wound-healing and anti-inflammatory activities.[3,4] Previous phytochemical reports on honey showed that this natural sweetener contains not less than one hundred phyto-substances. Potential pharmacologically active ingredients of honey include phenolic compounds, flavonoids and other phytochemicals which display a broad range of biological activities.[4-6] Actual health benefits derived from honey depend on the quality and purity of this natural substance.[7] Monofloral honey is a type of honey which has a high value in the marketplace because it has a distinctive flavor or other attribute due to its being predominantly from the nectar of one plant species.[8] Sidr monofloral honey is found in the desert areas of Yemen, Saudi Arabia and Pakistan's Potohar region.[9] Yemeni sidr honey is considered as the finest and of the best quality all

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DOI: *10.5530/pj.2015.3.3*

around the world and that is because of the Yemen's climate and environment are good habitats for making honey.[6]

Honey is traditionally used to treat dyspepsia and peptic ulcers.[2,10] *Helicobacter pylori*, the pathogenic agent in numerous conditions of digestive disorders and gastric ulcer was found to be susceptible to honey.[11] Also, honey is effective in treating bacterial gastroenteritis.[3,12] There are no scientific reports on the effectiveness of sidr monofloral honey to validate its traditional use on the cure and prevention of peptic ulcer. Many animal models of gastroulcerogenesis are used such as ethanol- or pylorus ligation-induced gastric ulcer.[13,14] Therefore, the current study was designed to investigate the effectiveness of sidr monofloral honey (SH) against ethanol-induced gastric ulcerogenesis.

MATERIALS AND METHODS

Materials

Pure sidr (*Ziziphus spina-christi* (L.) Desf.) honey was obtained from an exclusive honey shop in Jazan, Saudi Arabia.

Animals husbandry and caging

Sprague Dawley male rats (220 ± 20g) were obtained from the Experimental Animal House, Medical Research Centre, Jazan University, Jazan, Saudi Arabia. Animals were divided randomly into six groups (n=5). Food and water were provided throughout the experiment *ad libitum*. All animals received human care according to the criteria outlined in the "Guide for the Care and use of Laboratory Animals "prepared by the Medical Research Centre, Jazan University, Jazan, Saudi Arabia. Ethical approval was obtained in June 2012 [Ethic No MRC/2012/BMRU/132].

Ethanol-induced gastric ulcer

Rats were fasted for 48h prior to oral dosing. One hour before intragastric administration of 95% ethanol (5 ml/kg) animal treated as follow: group I treated with vehicle (5% Tween 80, v/v, 5 ml/kg.B.W.), group II omeprazole (20 mg/kg), group (III, IV & V) with honey (100, 250 and 500 mg/kg, respectively). One h after ethanol dosing, all animals were sacrificed under anesthesia (ketamine & xylazine) and their blood was collected.[15] Experimental design is depicted in Table 1.

Mucus content and pH of Gastric juice

Gastric juice of each animal was collected and centrifuged to measure the pH (meq/l) from the supernatant using the pH-meter. Weight of the gastric mucosa from the sedimentation using precise balance was obtained.[16]

Gross assessment of ulcer

Gastric ulcer appears as elongated bands of hemorrhagic lesions. The length (mm) and the width (mm) of each bands were measured using planimeter [(10×10 mm^2= ulcer area) under steromicroscope (1.8×)]. The area of each ulcer lesion was measured by counting he number of small squares (2×2) mm covering the length and width of each ulcer band. The sum of the areas of all lesions for each stomach was applied in the calculation of the ulcer area (UA) wherein the sum of small squares (4×1.8 = UA mm^2). The inhibition percentage (I %) was calculated by the following formula described in[17] with slight modifications:

$$\text{Inhibition percentage (I \%)} = \frac{[(UA\text{control} - UA\text{treated})}{UA\text{control}]} \times 100\%$$

Table 1: Effect of Sidr honey on ulcer index, gastric acidity and mucus weight of ethanol induced gastric ulcer

Animal group	pretreatment (5ml/kg)	pH of Gastric tissue	Mucus weight (g)	Ulcer Area (mm^2) (mean±S.E.M)	% Inhibition
I	Ulcer control	3.61±0.15	0.99±0.04	482.4±40.89	-
II	Omeprazole (20 mg/kg)	7.0±1.14	0.90±0.07	108±9.60	77.59
III	Sider Honey (100 mg/kg)	4.2±0.31	1.10±0.13	104±3.50	78.42
IV	Sider Honey (250 mg/kg)	4.9±0.02	1.37±0.02	69±1.52	85.69
V	Sider Honey (500 mg/kg)	6.5±0.01	1.41±0.01	36±5.36	92.53
VI	Normal control	5.06±0.09	0.258±0.06	-	-

Values are presented as mean ± SEM of five rats in each group. Groups with different alphabets are statistically different.

Microscopic evaluation using hematoxylin and eosin

For histopathological evaluation, a small fragment of each animal gastric ulcer was fixed with 10% buffered formalin solution. Formalin fixed and paraffin embedded (FFPE) sections of 4 μm were stained with hematoxylin and eosin (H &E) for light microscopy assessment.[18]

Immunohistochemistry

The FFPEs were cut to 4 μm thickness (Leica Rotation Microtome, Wetzlar, Germany). The tissue sections were then mounted on 3-aminopropyltrimethoxysilane (APES)-treated glass slides, dried overnight at ambient temperature and stored at -4ºC until used for immunohistochemistry. Deparaffinized FFPE gastric sections in buffer solutions were boiled in a microwave oven to retrieve the antigen. Endogenous peroxide was blocked with 3% (v/v) H_2O_2. After washing with Tris-buffered saline (pH 7.6) (TBS) containing 0.1% Tween-20, the sections were blocked with 0.01% d-biotin for pre-treatment with the heat-induced epitope retrieval method. The sections were further blocked with 3% bovine serum albumin for 1 h to minimise nonspecific antibody binding and then incubated with appropriate dilution of primary antibodies for 1 h at room temperature or overnight (~18 h) at 4°C. The control sections were not incubated with primary antibody. Immunostaining was performed using LSAB®2 System-HRP kit (DAKO, Carpinteria, USA) at room temperature according to manufacturer's instructions. The slides were treated with a biotin-conjugated secondary antibody for 30 min followed by 30 min incubation with peroxides-conjugated streptavidin at room temperature. At each step, the slides were washed with TBS. Peroxidase activities were detected after incubating the samples with 3, 3´-diaminobenzidine (DAB) by the appearance of a brown precipitate that is insoluble in alcohol. Finally, the sections were counterstained with hematoxylin. Specificity of staining was controlled by omission of primary antibody.

Immunohistochemical staining of Bax

Monoclonal anti-rat Bax antibodies (Santa Cruz Biotech., USA) were used at dilution of 1:200. The sections were pre-treated by boiling in 10 mM Tris buffer containing 1 mM EDTA (pH 6.0) for 30 min to unmask the antigen. The sections were then incubated with the primary antibody for 70 min at room temperature.

Phytochemical analysis

Total Phenolic (TPC) and Flavonoid (TFC) Content

The Folin-Ciocalteu method was utilized to examine the total phenolic content (TPC) of the honey. Values of TPC were estimated by comparing the absorbance of each sample with a standard response curve generated using gallic acid. The total flavonoid content was determined by using a colorimetric assay of aluminum tetrachloride as previously described. A calibration curve was calculated with quercetin, and the results were expressed as mg quercetin equivalents (CE)/100 g of honey.

Qualitative phytochemical screening

Sidr honey was subjected to phytochemical test using standard method.[19] Nuclear magnetic resonance (NMR) analysis was used to confirm the functional chemical groups in the honey.

Statistical analysis

All values were reported as mean ± S.E.M. Statistical significant differences between groups were assessed using one-way ANOVA followed by Tukey's *post hoc* multiple comparison test. A value of $P<0.05$ or lower was considered as a significant difference.

RESULTS AND DISCUSSION

Peptic ulcer is known to be one of the most common disturbing ailments. The utilization of herbal medicine and natural substances for the prevention and cure of human diseases is persistently and globally moving ahead. Phenolic compounds constitute the largest group of plants secondary metabolites and have attracted special attention due to their health-promoting characteristics.[20,21] Therefore, the current paper was an attempt to investigate the anti-ulcerogenic mechanism(s) of sider honey on ethanol-induced ulcer in rats. The model used in this study is oxidative stress-based mechanism.

Honey is a supersaturated liquid substance of sugars, mainly composed of glucose and fructose, containing also some nutrients such minerals, proteins, amino acids, enzymes, vitamins and polyphenols. Among polyphenols, flavonoids are the most plentiful and are directly related to its therapeutic properties. Honey certainly influences risk factors for heart diseases by inhibiting inflammation,

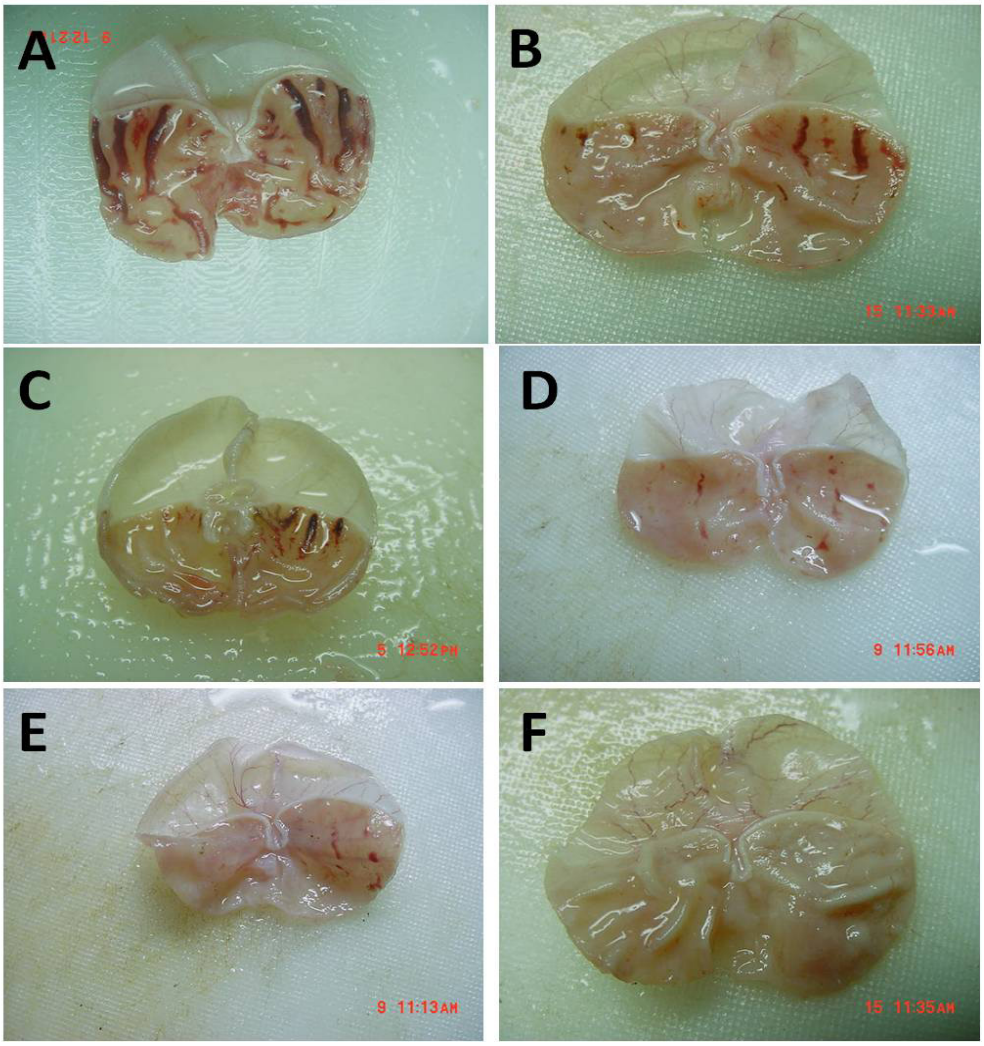

Figure 1: Gross evaluation of gastric wall from various animal groups.

Results showed that rats pre-treated with sidr honey at doses of 100, 250 & 500 mg/kg kg (photo 1C, 1D & 1E, respectively) and omeprazole (20 mg/kg, photo 1B) had considerably reduced areas of gastric ulcer formation compared to rats pre-treated with only vehicle (ulcer control group, Figure 1A) (Magnification: 1.8 X).

enhancing endothelial function, as well as the lipid profile, and increasing low-density lipoprotein resistance to oxidation. The evidence of the bioactivities of honey can be credited to its polyphenolic contents which, in turn, are regularly linked to its antioxidant and anti-inflammatory actions, as well as to its cardiovascular, antiproliferative and antimicrobial benefits.[22-24]

A variety of phenolic natural products have been documented for their anti-ulcerogenic activity with an excellent level of stomach protection. In addition to their action as gastroprotective, these antioxidant compounds can be an alternative for the cure of gastric ulcers.[25,26] Results of the current study showed that animals pre-treated with sider and omeprazole considerably reduced ulcer area formation compared to animal group pre-treated with only 5% Tween 80 (vehicle). Sider honey at doses of 100, 250 and 500 mg/kg b.w., significantly ($P<0.05$) inhibited ulcer formation by 78.42%, 85.69% and 92.53%, respectively, as depicted in Table 1 and Figure 1, respectively. As shown in Table 1, ulcer control group produced low mucus content of gastric mucosa, while various doses of sidr honey increased significantly ($P<0.05$) the mucus production. On the other hand, the animal groups pre-treated with sider honey showed increase in the pH of the gastric juice. The fact that acid gastric juice plays an important role in the genesis of peptic ulcer is supported by clinical, pathological and laboratory-based evidence.[27]

Microscopic evaluation was used to assess the anti-ulcer activities of sider honey. Histological observation of the ulcer control group pre-treated only with 5% Tween 80, showed highly extensive gastric lesion, submucosal edema and leucocytes infiltration. Pre-treatment with sider honey

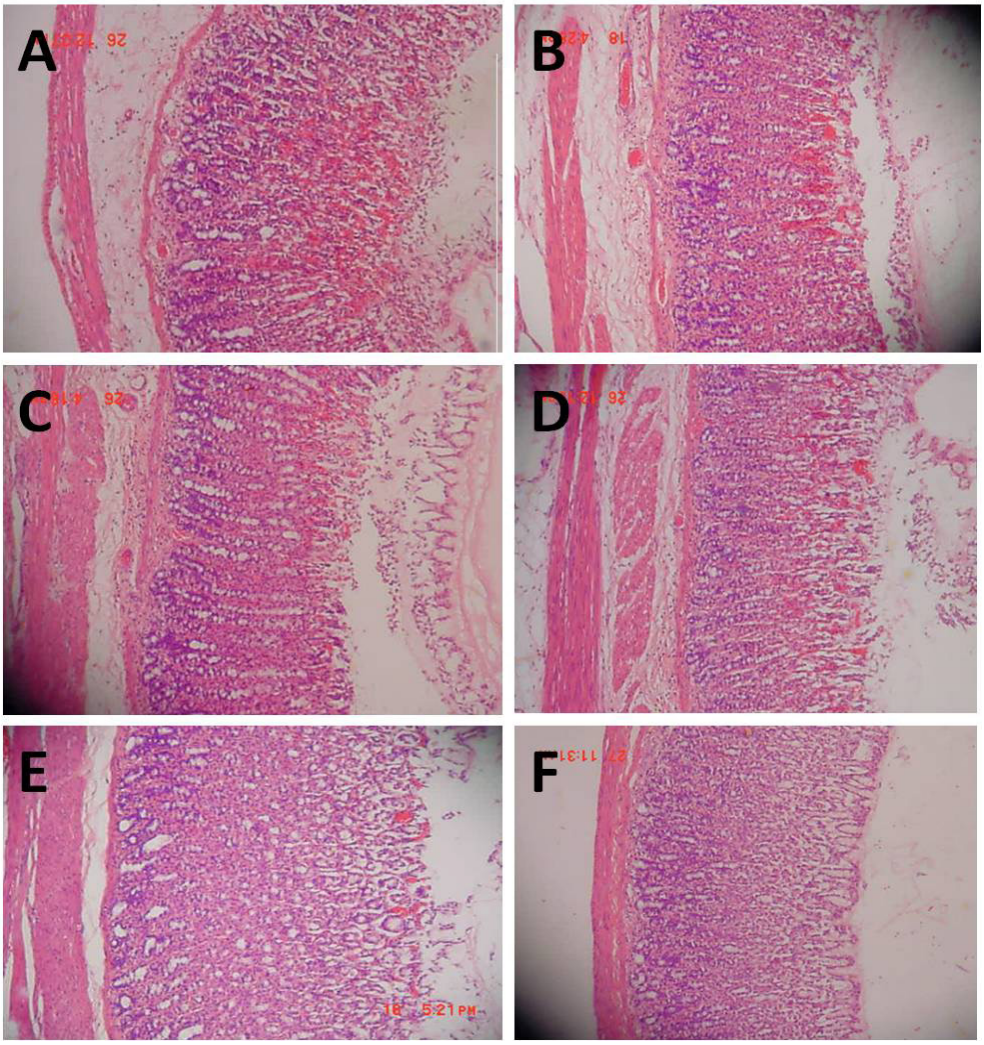

Figure 2: Histopathological evaluation.

Results showed that rats pre-treated with sidr honey at doses of 100, 250 & 500 mg/kg kg (photo 2C, 2D & 2E, respectively) and omeprazole (20 mg/kg, photo 2B) improved the histopathology compared to rats pre-treated with only vehicle (ulcer control group, Figure 2A) (H and E stain; 100x).

(100, 250 and 500 mg/kg) and omeprazole have relatively better protection as seen by decreasing ulcer area, reduction or complete absence of edema and leucocytes infiltration as shown in Figure 2. A recent study by Rafatullah demonstrated that the administration of Saudi Sidr honey prevented histomorphological lesions triggered by carbon tetrachloride in experimental rats. This protective effect of sidr honey was suggested to be through the presence of antioxidative compounds.[28] On the other hand, treatment of animals with sidr honey resulted in to the expansion of a substantial continuous PAS-positive mucous layer that lining the entire gastric mucosal surface noted as a bright-purple-stained area lining the mucosa as shown in Figure 3.

A previous report, on the role of mitochondrial energy charge in the etiology of ethanol-induced gastric mucosal injury, was published previously.[29] In response to increase level o free radicals, mitochondrial membrane leaking leads to the upregulation of apoptosis associated protein (Bax, proapoptotic factor). The pathological changes in mitochondria are positively related to ethanol exposure. As mentioned above, ethanol-induced gastric mucosal injury is related to oxidative stress, which disturbs the energy metabolism of mitochondria and plays a critical role in the pathogenesis of ethanol-induced gastric mucosal injury. The present study showed that pre-treatment with sidr honey led to the down-regulation of Bax as shown in Figure 4. These observations suggest the antiapoptotic effect of sidr honey against ethanol induced cell injury.

Phenolic compounds display a number of biological activities in the gastrointestinal tract, acting as antisecretory, cytoprotective, and antioxidant agents. The antioxidant activities of these natural ingredients have been widely

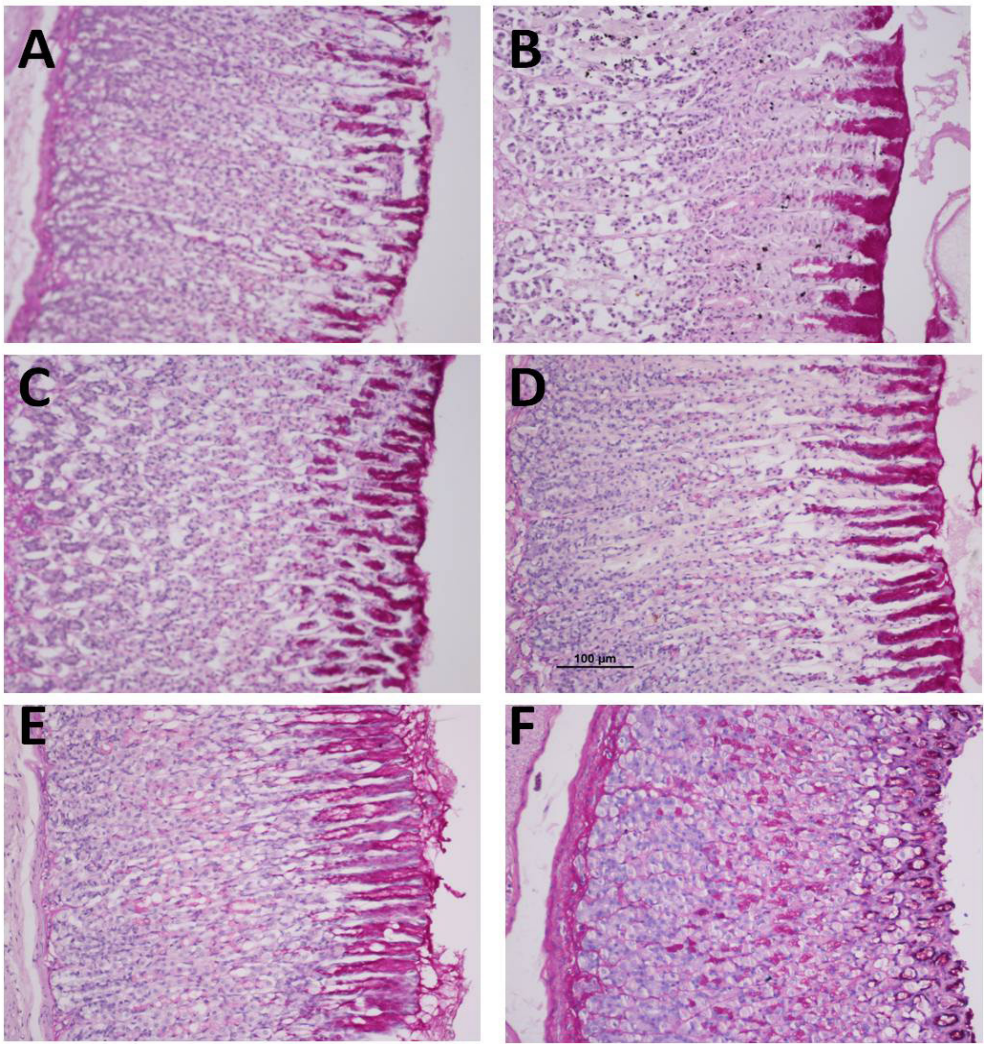

Figure 3: PAS staining for the evaluation of mucus production.

Results showed that rats pre-treated with 4-hydroxybenzoic acid at doses of 100, 250 & 500 mg/kg (photo 3C, 3D & 3E, respectively) and omeprazole (20 mg/kg, photo 3B) showed more PAS-positive mucus as compared to those pre-treated with only Tween80 (ulcer control group, Figure 3A) (100x).

Table 2: Effect of sidr honey of glutathione (GSH) and malondehyde (MDA) levels

Animal group	pretreatment (5ml/kg)	GSH	MDA (µmol/g tissue)
I	Ulcer control	0.7^{a}±0.10	28^{a}±0.18
II	Omeprazole (20 mg/kg)	8.3^{b}±1.29	2.4^{b}±0.26
III	Sider Honey (100 mg/kg)	4.9^{c}±0.38	2.9^{b}±0.13
IV	Sider Honey (250 mg/kg)	8.5^{b}±1.02	1.2^{c}±0.02
V	Sider Honey (500 mg/kg)	15.1^{c}±0.49	0.48^{d}±0.11
VI	Normal control	7.71^{b}±0.92	0.5^{d}±0.14

Values are presented as mean ± SEM of five rats in each group. Groups with different alphabets are statistically different.

investigated by many researchers, but it has become clear that their mechanisms of action go beyond the modulation of oxidative stress.[31-33] Deficiency of GSH within the body organs can lead to tissue injury and malfunction.[34] In the present investigation, GSH was remarkably (*P<0.01*) decreased in ulcer group than the normal group. The treatment of animals with the sider honey restored significantly (*P<0.01*) the GSH levels depletion caused by ethanol administration. These findings are in accordance with earlier reports as GSH levels were significantly depleted in gastric homogenate of rats, when exposed with ethanol.[35,36] Pretreatment of rats with sider honey replenished GSH level as compared with ethanl only treated animals, suggesting the ameliorative effects of sider honey. Moreover, ethanol was reported to induced

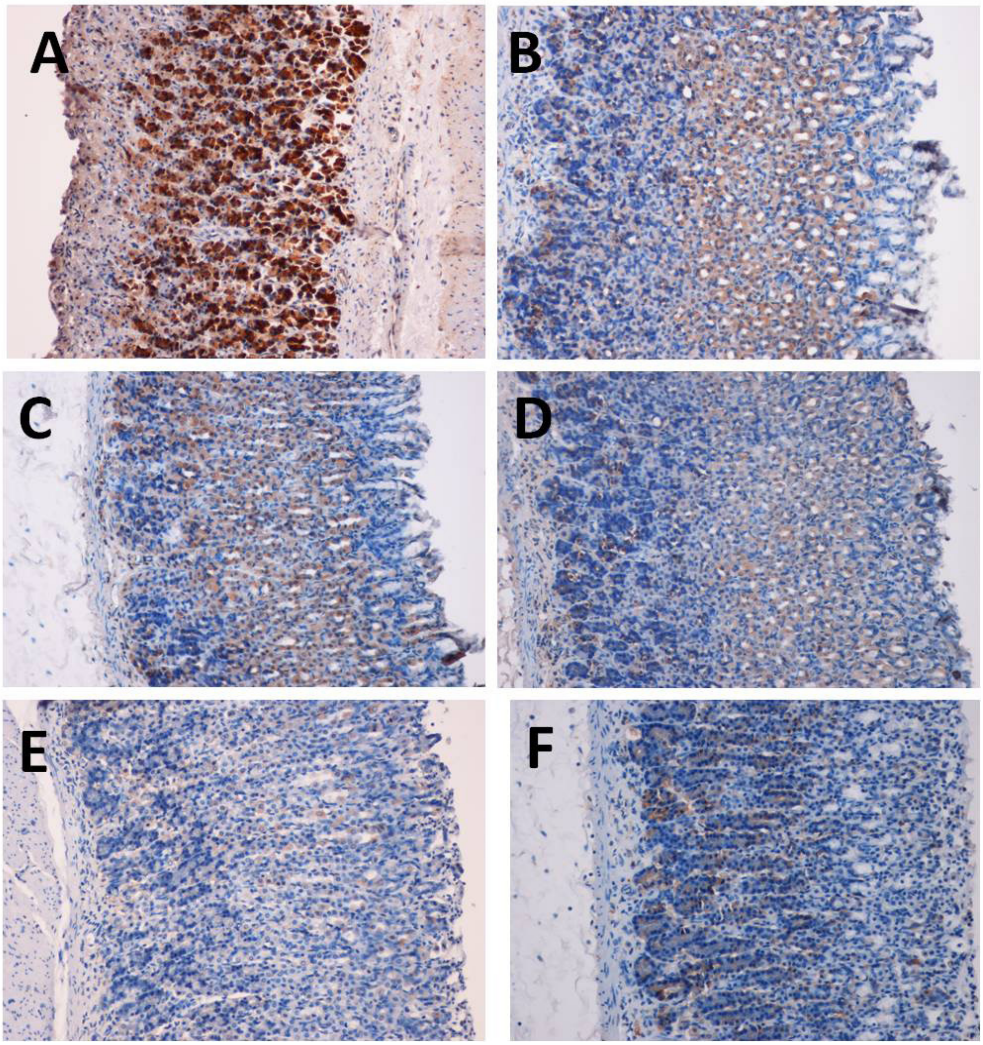

Figure 4: Immunohistochemical staining of rat gastric tissues with Bax primary antibody (100x).

lipid peroxidation in the gatric wall of induced animals.[37] In the present study, ulcer control group showed the highest malondialdehyde (MDA) level, an indicator of lipid peroxidation, than the other groups. Gastric MDA level significantly ($P<0.05$) dropped after sider hoeny administration. Results for MDA and GSH are showing n in Table 2. The antioxidant mechanism of Sidr honey was further analyzed using phytochemical studies. Saudi Sidr honey in this study, demonstrated high total phenolic and flavonoid contents (85.4 mg GAE/100 g and 42.5 QAE /100 g). The ^{1}H-NMR of sidr honey showed the presence of methyl, methylene and aromatic CH. However, analysis also showed the presence of hydroxyl group peak as well.

CONCLUSION

In conclusion, sidr honey remarkably and dose-dependently protects the gastric mucosa against ethanol-triggered damage. The antioxidant activity of this natural substance, through the induction of cellular antioxidant protection, is a pointer for scavenging the free radicals formed by ethanol. The current findings warrant further research for the introduction of sider honey as possible defensive and remedial agent for gastric ulcer that caused by different etiologies. Authors are currently analyzing the phytochemical content of sidr honey.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research was graciously supported by the Scientific Chair for Prophetic Medicine and Scientific Miracle (MABL Chair).

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Evaluation of Phytoconstituents of *Bryonopsis laciniosa* fruit by UV-Visible Spectroscopy and FTIR analysis

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ABSTRACT

Objectives : To Investigate the phytochemicals present in *Bryonopsis laciniosa* fruit. **Methodology:** Qualitative, Quantitative screening, Compound Identification by UV-Visible method and identification of functional group of the active chemical components were followed by standard procedures. **Result :** The results showed the presence of phytonutrients like reducing sugar, terpenoids, triterpenoids, aminoacids, anthroquinone, polyphenols, glycosides, anthocyanins, tannins, coumarins, emodins, saponins, total alkaloids, total flavonoids, lignin and serpentine. These substances may be responsible for the health related properties of the plant which are based on antioxidant,anticancer, antipyretic, antiaphoretic, antidiabetic, anti-inflammation, antiheamatisum, antimicrobial and antiviral activity. **Conclusion:** This study supports the popular use of *Bryonopsis laciniosa* fruit in preparation of various pharmaceutical formulations for human welfare.

Key words: *Bryonia laciniosa,* FTIR, Phytochemical screening, Phytonutrients, UV-Vis.

INTRODUCTION

India is sitting on a gold mine of well-recorded and traditionally well-practiced knowledge of herbal medicines, therefore, any scientific data on such plant derivatives could be of clinical importance Medicinal plants are the richest bio-resource of drugs of traditional system of medicine, modern medicines, nutraceuticals intermediates and chemical entities for synthetic drugs Medicinal plants are of great importance to the health of individuals and communities They are generally used in traditional medicine for the treatment of many ailments[1]

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DOI: *10.5530/pj.2015.3.4*

Many plants contain a variety of phytopharmaceuticals, which have found very important applications in the fields of agriculture, human and veterinary medicine. The use of plants as therapeutic agents in addition to being used as food is age long and there is a great awareness in the use and significance of these medicinal floras (WHO 2002). This has led to intensified efforts on the documentation of medicinal plants.[2] Phytochemicals are natural bioactive compounds found in plants that functions along with dietary fiber and nutrients which protect against various diseases. Vegetables, fruits and nuts are the rich sources of phytochemicals, help in slow aging and reduce the risk of many diseases, including diabetes, heart diseases, infections, cancer etc.[3] Some important phytochemicals are alkaloids, flavonoids, tannins, saponins that posses antifungal,antib acterial,anticancer,anti-inflammatory.[4] The phytochemical analysis of the plants is very important commercially and has great interest in pharmaceutical companies for the production of the new drug for curing of various diseases The knowledge of the chemical constituents of plants

would further be valuable in discovering the actual value of folk medicines.[5]

Bryonopsis laciniosa (*Linn)* is annual slender plant in the cucurbitaceae family native throughout India from the Himalayas to Ceylon, tropical Africa, Australia. *Bryonopsis laciniosa* is a highly valuable medicinal cucurbit commonly known as lollipop climber and it is called as "Shivlingi" in India. It is one of the most versatile medicinal plant having a wide spectrum of Biological activity[6]

Stem is much branched, slender, grooved and glabrous. Tendrils are slender, scabrous above, smooth, margin denticulate. Flowers monoeicious, often male and female clustered together. Pedicels shorter in male flowers. Fruits barriers, spherical yellowish-green or green-white, 6-stipped, 12-17 mm thick up to 2 cm across. Seeds ovoid, with thickened, corrugated, margins. It is bitter and aperients, and is considered to have tonic properties.[6] Plant flowers and fruits during the period from August to December

Bryonopsis laciniosa leaves and fruits are cooked as vegetables.[7] Leaves and seeds are anti inflammatory and febrifuge. They are used to treat flatulence, fever and reduce inflammation. The seeds are used in Homeopathy and Ayurveda as a tonic for females and they rejuvenate female reproductive system and promotes conception of child. In males the seeds promote spermatogenesis and increase sperm count. The seeds are also used for snake bite. Seeds are antibacterial and anti-fungal. In Homeopathy, a tincture made from the roots are the lollipop plant is prescribed for the treatment of inflammation of uterus, vaginal disorders and other urinary genital proplems.[8] A juice made from the leaves can be applied for pains and joints. Whole plant is used to treat ailments such as asthma, cough and bronchitis .Fruits are used as aphrodisiac, tonic. Sharp, cutting, lancinating or tearing pain, inflammation with muscular tension are cured by this plant The aim of this study is to determine the phytochemicals, in the *Bryonopsis laciniosa (Linn)* fruit, which may provide an insight in its use in traditional medicine.

MATERIALS AND METHODS

Collection of *Bryonopsis laciniosa* fruit

Bryonopsis laciniosa (Linn) is an annual scan dent herb and is widely spread in india The fruits ware collected from Ramanathapuram Distrct. (Identification: These samples were authenticated by Dr. V. Ramachandran. Associate professor, Department of Botany, Bharathiyar University, Coimbatore. A Voucher specimen (Number:BU/Dept BOT/Bl/16.06.2014) has been deposited at the Herbarium, Bharathiyar University, Coimbatore, Tamil Nadu, India. Each specimen was washed under running tap water, labeled, weighed and annotated with the date of collection. Each specimen dried at 37^{0}C for 15 days, powdered and stored in air tight container.

Preparation of Fruit extract

2 gm of dried finely powdered fruit was taken in a beaker. 30 ml of distilled water and 70 ml of methanol was added. The mixture was shacked by continuous stirring at room temperature for 30 minutes and kept for 2 days. Then the solvent was allowed to evaporate and the extract was used for the phytochemical analysis.

Phytochemical Screening

Preliminary phytochemical tests were carried out in the ethanolic extract of *Bryonopsis laciniosa* fruit using standardized procedures to identify the constituents as described.[9]

Quantitative analysis of secondary metabolites by HPLC Analysis

Sample preparation

The extraction was carried out using 2 ml of fermented broth with 50 mL of 95% ethanol under 80 KHz, 45°C in ultrasonic extraction device for 30 min, repeated twice. The extract was collected and filtered; the filtrate was dried at 50°C under reduced pressure in a rotary evaporator. The dried crude extract was dissolved in 100 ml mobile phases. After filtering through a filter paper and 0.45 mm membrane filter (Millipore), the extract was injected into HPLC.

HPLC conditions

Samples were analyzed using an RP-HPLC method,[10] Shimadzu Corp., Kyoto, consisting of a LC-10ATVp pump, SCL 10A system controller and a variable Shimadzu SPD- 10ATVp UV VIS detector and a loop injector with a loop size of 20 µl. The peak area was calculated by a CLASSVP software. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250×4.6 mm i.d., particle size 5 µm, Luna 5 µ C-18; phenomenex, Torrance, CA, USA) at 25°C. The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed, using dual pumping system, by varying the

proportion of solvent a [water-acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 50% in 4 minutes and subsequently increased to 80% in 10 minutes at a flow rate of 1.0 mL/min. Detection wavelength was 280 nm.

UV visible and FTIR Spectroscopic analysis

The extracts were examined under visible and UV light for proximate analysis. For UV and FTIR spectrophotometer analysis, the extracts were centrifuged at 3000 rpm for 10 minutes and filtered through Whatmann No. 1 filter paper by using a high pressure vacuum pump. The sample is diluted to 1:10 with the same solvent. The extracts were scanned in the wavelength ranging from 260-900 nm using Perkin Elmer Spectrophotometer and the characteristic peaks were detected. FTIR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks ranging from 400-4000 cm^{-1} and their functional groups. The peak values of the UV and FTIR were recorded. Each and every analysis was repeated twice for the spectrum confirmation.[11]

RESULTS AND DISCUSSION

In this present study the *Bryonopsis laciniosa(Linn)* fruit extract was Subjected to phytochemical screening and it is represented in Table 1. Saponins, flavonoids are found to be abundant while terpenoids, triterpenoids, proteins, anthocyanins, coumarins are in moderate amount. Alkaloids, reducing sugar, amino acids, tannins, polyphenols, glycosides, emodins are present in trace amount. Selectively total alkaloids, total flavonoids, tannin, lignin, serpentine and glycosides were estimated quantitatively and given in Table 2. The fruit are rich in the flavonoid content when compared to glycosides, alkaloids, tannins, lignins and serpentine.

The fruit of *Bryonopsis laciniosa* appear to be rich in secondary metabolites, widely used in traditional medicine to combact and cure the various ailments. Flavonoids are found to be abundant in the fruit and they protect against allergies, Platelet aggregation, microbial infections, Ulcers, hepatotoxins and tumors.[12] Flavonoids reduce the risk of estrogen induced cancers by interfering with the enzymes that produce estrogen.[13] They act as powerful protective agent against gastrointestinal infections, inflammations, Odema and inhibit the synthesis of Prostaglandin E2, F2 and thromboxane B2. They are powerful antioxidants and free radical scavengers, which prevent oxidative cell damage, and have strong anticancer activity They also inhibit microbes which are resistant to antibiotics. Presence of Saponins are responsible for the, foaming activity and cell membrane permeabilizing activity. Especially Saponins have haemolytic property, induce cytotoxic effect, expectorant action, antitumor, antimutagenic activities and can reduce the risk of cancers by preventing the cancer cells from growing. It also used to stop bleeding and in treating wounds.[14] Traditionaly saponins are used as detergents, foaming, surface active agents, pesticides, molluscides and have relationship with oxytocin which controls the onset of labor of women and the subsequent release of milk.[15] Saponin have the ability to modulate the cell mediated immune system as well as enhance antibody production.[16] Terpenoids posses membrane distruption and inhibitory effect against fungi and bacteria and also have inflammatory, analgesic, anticancer, antimicrobial, antiviral, anti ulcer, hepaticidal and antitumor activities.[17] Anthocyanins help the immune system and protect the body against influenza virus. Various studies have been

Table 1: Preliminary phytochemical Screening of *Bryonopsis laciniosa* fruit extract

Name of the Compound	Observation
Tannins	+
Phlobatannins	---
Saponin	+ + +
Flavonoids	+ + +
Steroids	---
Terpenoids	+ +
Triterpenoids	+ +
Alkaloids	+
Carbohydrates	+
Amino acids	+
Anthroquinones	---
Polyphenols	+
glycosides	+
Proteins	+ +
Anthocyanins	+ +
Coumarins	+ +
Emodins	+

(+) Presence; (--) Absence ++ = Medium, +++ = High concentrations.

Table 2: Quantitative analysis of Phytochemicals of *Bryonopsis laciniosa* fruit extract

Name of the Compound (mg Kg-1)	Observation
Total Alkaloids	0.86 ± 0.05
Total Flavonoids	2.36 ± 0.16
Tannin	0.35 ± 0.02
Lignin	0.42± 0.02
Glycoside	0.06 ±0.01
Serpentine	0.13 ±0.01

Table 3: UV-VIS Peak Values of Extract of *Bryonopsis laciniosa* fruit

Wave length (nm)	Absorption peak
208.04	2.76
271.94	0.72
881.96	0.04

proved that coumarins have strong antioxidant effect due to its ability to show both antimutagenic as well as anticarcinogen effect Many coumarin derivatives act as free radical scavengers[18] Non toxicity and high activity of several Coumarins was observed in the inhibition of carcinogenesis produced by benzo[*a*]pyrene.[19] Generally Coumarins are highly potent anti inflammatory agent and directed against cell-adhesion molecule and they play as effective anticoagulants by inhibiting the function of Vitamin K which is essential for prothrombin biosynthesis.[20] Alkaloids posses plasmolytic, anticholinergic, analgesic, stimulants, antimalarials and anesthetic activity and reduces the fever and headache.[21] Phenols are highly effective anticoagulants, antioxidants, immune enhancers, hormone modulators, and they modify the prostaglandin pathways, protect platelets from clumping and inhibits the enzymes which stimulates the inflammation.[22] Saponins and Glycosides shows antimicrobial activity.[23] Glycosides are cardio protective and used to treat the cardiac arrhythmia and congestive heart failure Tannins are moderately present and posses haemostatic activity, potential biological antioxidant, anti diarrheal, anti hemorrhoidal, antiviral, antiparasitic,[24] antibacterial, antifungal, proton precipitating agent and effective metal ion chelator.[25] Apart from this tannins posses astringency property i.e. faster the healing of wounds and inflamed mucous membrane. The potential anti mutagenic and anti carcinogenic activity has been related to their anti oxidative property, which is important in protecting cellular damage including lipid peroxidation. Moderate amount of polyphenols posse's cellular support and form the integral part of the cell wall. They are anti apoptotic, anti aging, anti carcinogenic, anti inflammatory, anti atherosclerotic, cardiovascular protective and prevent from oxidative stress as well as inhibition of angiogenesis and cell proliferation.[26] Emodins has anti angiogenetic effect[27] Plenty of pharmaceutical studies indicates that emodins have anti inflammatory, anticancer and antimicrobial activity anti-cancer, anti-microbial and anti-inflammatory effects.[28]

The UV-Visible spectra were performed to identify the compounds containing σ- bonds, π-bonds, and lone pair of electrons, chromophores and aromatic rings The profile showed the peaks at 208.04, 271.94 and 881.96 nm with the absorption 2.76,0.72 and 0.04 respectively Table 3 and Figure 1. The result confirms the occurrence of peaks at 208-881 nm reveals that the absorption bands are due to the presence of flavonoids, phenol and its derivatives in the bryonopsis laciniosa fruit.[29]

FTIR Spectrum identified the functional group of the active chemical components present in the fruit based on the peak value in the region of infra red radiation. When the fruit extract was passed in to the FTIR, the functional group of the components was separated based on its ratio. The peak values and the functional groups were represented in

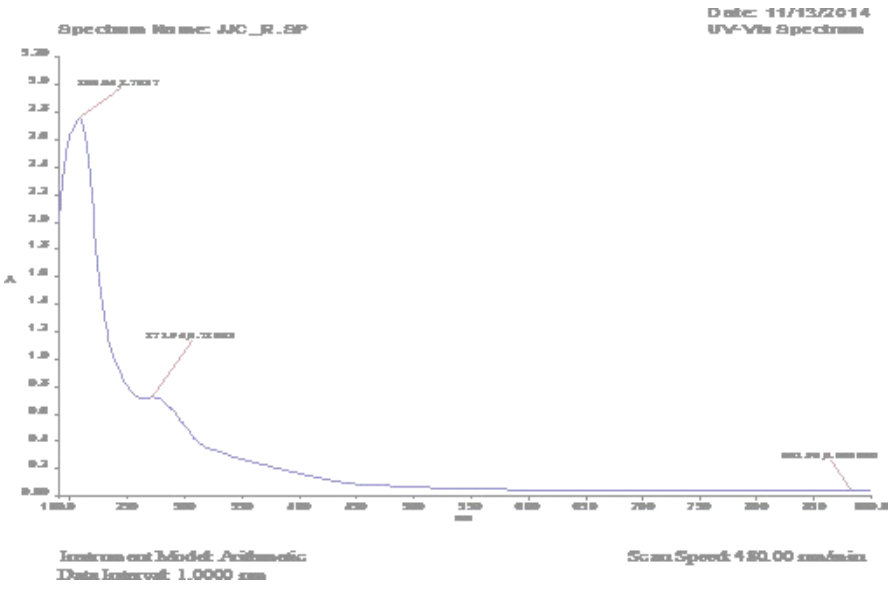

Figure 1: UV-Vis Spectral analysis of *Bryonopsis laciniosa* fruit extract

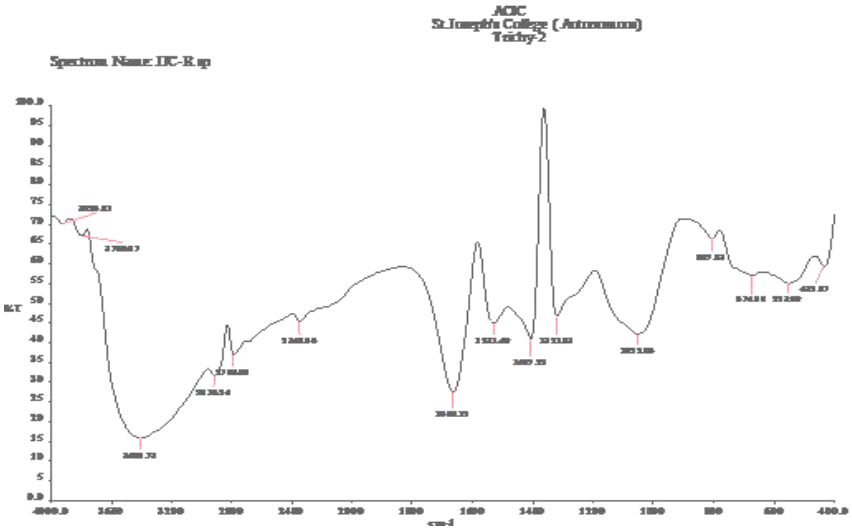

Figure 2: FTIR analysis of *Bryonopsis laciniosa* fruit extract

Table 4: FTIR Peak Values of Extract of *Bryonopsis laciniosa* fruit

Peak Values	Functional groups
3926.81	Unknown
3796.97	Unknown
3409.73	Unknown
2916.24	Alkanes
2788.08	Deuterated R- OH
2349.66	Unknown
1665.12	Alkenes
1531.40	Ketones
1407.22	Aromatics
1321.61	Alcohols,carboxylic acids,esters,ethers
1052.66	Aliphatic Amines
807.83	Aromatic P-substituted
674.88	Halogen Compound
553.90	Sulfur compounds
432.87	Unknown

extract was passed in to the FTIR, the functional group of the components was separated based on its ratio. The peak values and the functional groups were represented in Table 4 and Figure 2. The results of the FTIR confirmed the presence of Alkenes, Alkenes, Ketones, Aromatics, Carboxylic acids, Esters, Ethers, Aliphatic amines, Halogen compounds, Sulphur compounds and Aromatic P substituted compounds.

CONCLUSION

The results obtained in present study reveals that *Bryonopsis laciniosa* fruit extract posses, various, phytochemical,

Table 4 and Figure 2. The results of the FTIR confirmed the presence of Alkenes, Alkenes, Ketones, Aromatics, Carboxylic acids, Esters, Ethers, Aliphatic amines, Halogen compounds, Sulphur compounds and Aromatic P substituted compounds.

CONCLUSION

The results obtained in present study reveals that *Bryonopsis laciniosa* fruit extract posses, various, phytochemical, constituents like Flavonoids, saponins, terpinoids, triterpinoids, reducing, sugar, proteins, anthocyanins, tannins, polyphenols, emodins, glycosides, coumarone, lignin and serpentine. Therefore screening intimates, presence of many bioactive chemical constituents which act as anti inflammatory, anticancer, antimicrobial, antioxidants, antidiarrheal, antihemorrhoidal agents. It is suggested that further work should be carried out to isolate, purify and possibly characterize the active constituents responsible for the activity of the plant. Scientific validation is necessary before being put in to practice.

CONFLICTS OF INTEREST

Authors do not have any conflict of interest.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. S.Velavan, Director, Harman Institute of Science Education and Research, Thanjavur, Tamil Nadu for providing facilities to carry out

the work. Grateful thanks are extended to Mr. N. Kannan, Executive Officer, and District Water Shed development agency. And Mr.Vincent Sahayaraj, Lab administrator, St.Joseph College Tiruchirappalli for helping in technical part of the work

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Jackfruit (*Artocarpus heterophyllus*) seed extract exhibits fibrino(geno)lytic activity

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ABSTRACT

Objective: The current study assesses the fibrinogen and fibrin clot hydrolyzing activities of aqueous seed extract of Jackfruit (AqSEJ). **Methods:** The protein banding pattern of AqSEJ (100 μg) was analyzed on SDS-PAGE. The proteolytic activity of AqSEJ was confirmed by spectrophotometer and zymography experiments. Fibrinogen, fibrin and plasma protein hydrolyzing activities of AqSEJ were analyzed on SDS-PAGE under reduced conditions. Plasminogen activation and indirect hemolytic activities was analyzed using spectrophotometer. The non-toxic property of AqSEJ was tested by edema, hemorrhage in experimental mice. **Results:** AqSEJ exhibited proteolytic activity and the specific activity was found to be 1.04 units/mg/min. Furthermore, AqSEJ non-specifically hydrolyzed Aα, followed by Bβ and γ chains of human fibrinogen and specifically hydrolyzed α polymer and α chain of partially cross linked human fibrin clot without affecting β chain and γ-γ dimer even up to the tested dose of 30 μg for the incubation period of 8 hours. Importantly, AqSEJ did not hydrolyze other plasma proteins and devoid of plasminogen activation property. The proteolytic activity of AqSEJ was completely neutralized by PMSF and IAA, while EDTA, EGTA, 1,10-Phenanthroline did not, suggesting the presence of serine and cysteine family proteases. Moreover, AqSEJ did not cause edema and hemorrhage in experimental mice up to the tested dose of 200 μg and non-toxic to RBC cells. **Conclusion:** AqSEJ hydrolyzes fibrinogen and fibrin clot and non-toxic in nature. Hence, this work showcases the potential applications of Jack fruit seed proteases in the treatment of thrombotic disorders.

Key words: Fibrino(geno)lysis, Hemostasis, Jackfruit, Moraceae, Non-hemorrhagic, Serine/cysteine protease.

INTRODUCTION

Proteases are ubiquitous hydrolytic enzymes involved in several physiological reactions including digestion, blood coagulation cascade, compliment system, apoptosis pathways and invertebrates prophenoloxidase activating cascade. Proteases have been extensively used in food industry, leather industry and pharmaceutical industries. In addition, proteolytic enzymes could be used as therapeutic agents to treat cancer (L-asparaginase), thrombotic disorder (nattokinase) and wound healing (papain).[1-4] The Jackfruit is popularly known as a seasonal fruit that belongs to the family of Moraceae and genus *Artocarpus* Lam. Though it is native to India, it is widely distributed especially in the tropical areas of East Asia and South America. Generally, the fruit part will be consumed due to its delectability; however, many times the seed will be discarded. While, in the southern part of India seeds are consumed as food. Jackfruit seeds are rich sources of both macro and micronutrients.[5-8] According to the recent studies Jackfruit seeds were found to contain anticancer, antihypertensive, antiulcer, antioxidant, antifungal, and antimicrobial

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DOI: *10.5530/pj.2015.3.5*

properties.[9,10] In addition, the Jackfruit seeds are beneficial in blood purification and curing pancreatic ailments.[11] The large array of therapeutic applications of Jackfruit seeds is attributed to the major biochemical constituents such as enzymes/peptides/proteins and phytochemicals. Jackfruit seeds are reported to contain the highest amount of trypsin, chymotrypsin, elastase inhibitors and lectin family glycoprotein's (Jacalin).[12-14] For instance, Jacalin (immuno modulatory glycoprotein) is one of the most important lectin characterized so far from Jackfruit seeds, however, the proteolytic enzymes were least explored.[15] However Siritapetawee *et al* (2012) reported on antimicrobial protease (AMP48) and novel serine protease having fibrino (geno) lytic activities from *Artocarpus heterophyllus* latex. Therefore, the current study reports the fibrinogen and fibrin hydrolyzing properties of Jackfruit seed aqueous extract (AqSEJ).

MATERIALS AND METHODS

Materials

Fat free casein, phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra acetic acid (EDTA), ethylene glycol-N, N, N', N'- tetra acetic acid (EGTA), iodoacetic acid (IAA), 1,10-Phenanthroline were purchased from Sigma Chemicals Co. (St.Louis, USA). Molecular weight markers were from Bangalore Genei Private limited, India. Human plasma fibrinogen was purchased from Sigma Chemicals Co. (St Louis, USA). All other chemicals used were of analytical grade. Fresh human blood was collected from healthy donors for the platelet rich plasma (PRP). Swiss albino mice weighing 20-25 g (from the central animal house facility, Department of Zoology, University of Mysore, Mysore, India) were used for pharmacological studies. Animal care and handling compliance with the National Regulations for Animal Research. The animal experiment was carried out after reviewing the protocol by the animal ethical committees (UOM/IAEC/06/2011 dated 12/07/2011) of the University.

Plant material

Artocarpus heterophyllus Lam (Jackfruit) seeds were obtained in the month of April and May from Pittenahalli village, Tumkur district and identified by Dr. P.Sharanappa, University of Mysore. A voucher specimen (PS/52/18 FEB 2012) has been preserved at the herbarium of Bioscience Department, University of Mysore, Hassan PG campus, Hassan for future allusion.

Preparation of Jackfruit seed extract

Brown coat was removed from the Jackfruit seeds thoroughly chopped and homogenized using double distilled water and centrifuged at 2000 g for 20 min at 4°C. The supernatant was collected and proteins were precipitated using 30% of ammonium sulphate. The precipitated protein sample was again centrifuged at 3,500 g for 20 min; pellet was dissolved and dialyzed overnight. The protein sample obtained was stored at -20°C until use. This extracted protein sample was used throughout the study and referred as Jackfruit seed aqueous extract (AqSEJ).

Protein estimation

Protein concentration was determined as described,[16] using Bovine serum albumin (BSA) as standards.

Proteolytic activity

Colorimetric estimation

Proteolytic activity was assayed as described.[17] Briefly, fat free casein (2%) was incubated with AqSEJ (60 µg) for 2.5 hours at 37°C. Sodium carbonate (0.4 M) and Folin-Ciocalteu reagent were added sequentially and the color development was read at 660 nm. One unit of enzyme activity was defined as the amount of the enzyme required to cause an increase in OD of 0.01 at 660 nm/min at 37°C. The specific activity was expressed as the units/min/mg of protein. For inhibition studies, similar reaction was performed independently after preincubating the AqSEJ (60 µg) for 30 min with 5 mM each of EDTA, 1,10-Phenanthroline, EGTA, PMSF and IAA.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Zymogram

SDS-PAGE (10%) and zymogram were carried as described previously.[18] Breifly, the AqSEJ (100 µg) prepared under reducing and non-reducing condition was used for SDS-PAGE. For zymogram, the AqSEJ (60 µg) prepared under non-reduced condition was loaded on to polymerized 2% casein/gelatin in resolving gel. After electrophoresis, protein bands were detected using Coomassie brilliant blue R-250. Molecular weight standards from 200 to 14.3 kDa were used to compare the protein bands of AqSEJ. For zymogram, after electrophoresis gels were washed with 10 mM sodium phosphate buffer containing 2.5% of Triton X-100 with constant agitation for 1 hour to remove SDS. The gel was incubated overnight at 37°C in Tris–HCl buffer (50 mM) pH 7.6 containing 10 mM $CaCl_2$ and 150

mM NaCl. Gel was then stained to observe the translucent activity bands.

Periodic acid-Schiff (PAS) staining

SDS-PAGE was carried out as mentioned above. PAS staining was done as described.[19] After the electrophoresis gel was fixed in 7.5% acetic acid solution and stored at room temperature for 1 hour. Then gel was washed with 1% nitric acid solution and kept in 0.2% aqueous periodic acid solution and stored at 4°C for 45 min. Then the gel was placed in Schiff's reagent at 4°C for 24 hours and was destained using 10% acetic acid to visualize pink color band.

Fibrinogenolytic activity

Fibrinogenolytic activity was determined as described previously.[20] AqSEJ (0-20 µg) was incubated with the human plasma fibrinogen (50 µg) in a total volume 40 µL of 10 mM Tris-HCl buffer pH 7.4 for 4 hours at 37°C. After an incubation period, reaction was terminated by adding 20 µL denaturing buffer containing 1 M urea, 4 % SDS and 4 % β-mercaptoethanol. It was then analyzed by 10% SDS-PAGE. For inhibition studies, AqSEJ (20 µg) was preincubated for about 15 min with 5 mM each of PMSF, IAA, EDTA and 1,10-Phenanthroline.

Fibrinolytic activity

Colorimetric estimation

Briefly, 100 µL of citrated human plasma was mixed with 30 µL of 0.2 M $CaCl_2$ and incubated for 2 hours at 37°C. The clot obtained was washed thoroughly for 5-6 times with phosphate buffer saline (PBS) and suspended in 400 µL of 0.2 M Tris–HCl buffer pH 8.5. The reaction was initiated by adding varied concentration of AqSEJ (0-30 µg) in 100 µL of saline and incubated for 2 hours 30 min at 37°C. The undigested clot was precipitated by adding 750 µL of 0.44 M trichloroacetic acid (TCA) and allowed to stand for 30 min and centrifuged for 15 min at 1,500 g. The aliquots of 0.5 mLsupernatant were transferred to clean glass tubes and followed by the addition of 1.25 mL of 0.4 M sodium carbonate and 0.25 mL of 1:3 diluted Folin-Ciocalteu's phenol (FC) reagent. The color developed was read at 660 nm after allowed to stand for 30 min. One unit of activity is defined as the amount of enzyme required to increase in absorbance of 0.01 at 660 nm/hour at 37°C.

Protein banding pattern on SDS-PAGE

Fibrinolytic activity was determined as described.[21] Tri-sodium citrate (3.2%) treated blood was centrifuged for 15 min at 500 g to separate platelet poor plasma. Plasma (100 µL) was mixed with an equal volume of 0.25 M $CaCl_2$ for 15 min at 37°C to get the soft fibrin clot. The fibrin clot was washed thoroughly 5–6 times with phosphate buffered saline (PBS) and suspended and incubated with AqSEJ (0–30 µg) in a final volume of 40 µL 10 mM Tris–HCl buffer pH 7.4 at 37°C for 8 hours. For inhibition studies, AqSEJ (30 µg) was pre incubated for about 15 min with 5 mM each of PMSF and IAA. The reaction was stopped by adding 20 µL of sample buffer containing 4% SDS, 1M urea and 4% β-mercaptoethanol. The samples were kept in a boiling water bath for 5 min and centrifuged to settle the particulate of the plasma clot. An aliquot of 20 µL supernatant was analyzed in 7.5% SDS-PAGE for fibrin degradation.

Plasminogen activation

The plasminogen activation was assayed as described.[22] Briefly, the samples of 1 mg of plasminogen contaminated fibrinogen or 20 µL of citrated human plasma with 200 IU of streptokinase with 50 µg of AqSEJ and 200 IU of streptokinase alone were independently mixed with 500 µL of azocasein and incubated for 3 hours at 37°C. The reaction was terminated by adding 400 µL of 25% trichloroacetic acid. The supernatant (600 µL) was diluted with an equal volume 0.5 N NaOH and absorbance was read at 440 nm. One unit of activity was defined as the amount of enzyme yielding an increase in absorbance of 0.01/hour at 440 nm.

Indirect hemolytic activity

Indirect hemolytic activity was determined as described previously[23] using washed human erythrocytes in presence of 0-200 µg of AqSEJ. The amount of hemoglobin released in the supernatant was measured at 540 nm.

Degradation of human plasma proteins

Degradation of human plasma protein was assayed as described previously.[24] The AqSEJ (0-50 µg) was incubated with the 100 µg of plasma proteins for 12 hours at 37°C in a reaction volume of 40 µL 10 mM Tris–HCl buffer pH 7.4 containing 10 mM NaCl and 0.05 % sodium azide. The reaction was terminated by the addition of 20 µL denaturing buffer containing 4% SDS and boiled for 5 min and analyzed on 7.5% SDS-PAGE under non-reduced condition.

Edema inducing activity

Edema inducing activity was done as described earlier.[25]

The groups of five mice were injected separately in to the right foot pads with different doses (0-200 µg) of AqSEJ in 20 µL saline. The left foot pads received 20 µL saline alone and served as controls. After 1 hour mice were anaesthetized by diethyl ether inhalation. Hind limbs were removed at the ankle joint and weighed. Weight increase was calculated as the edema ratio, which equals the weight of an edematous leg×100/weight of a normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120 %.

Hemorrhagic activity

Hemorrhagic activity was assayed as described.[26] A different concentration of AqSEJ (0-200 µg) was injected (intradermal) independently into the groups of five mice in 30 µL saline. The groups receiving saline alone served as a negative control and the group receiving Daboia resselli venom (2 MHD) served as the positive control. After 3 hours, mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage against saline injected control mice. The diameter of hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10 mm of hemorrhage in diameter.

Statistical Analysis

The data are presented as mean s ± S.E.M of at least five animals in each group. Difference among the data were determined by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT).Data were considered different at $P < 0.01$.

RESULTS AND DISCUSSION

Characterization of proteins and identification of proteolytic activity of AqSEJ

AqSEJ exhibited varied protein bands in the broad molecular weight range from 200 kDa to 14.3 kDa under both reduced and non-reduced conditions (Figure 1a). AqSEJ hydrolyzed casein with the specific activity of 1.04 units/mg/min suggesting the proteolytic activity. The proteolytic activity of AqSEJ was further confirmed by casein and gelatin zymogram, it showed two similar translucent activity bands in the higher molecular weight region (Figure1b & c). In order to identify the nature of protease present in the AqSEJ inhibition studies were carried out using specific protease inhibitors such as PMSF, IAA, EDTA, EGTA and 1,10-Phenanthroline. Interestingly, proteolytic activity of AqSEJ was completely neutralized by PMSF, a serine protease inhibitor and IAA a cysteine protease inhibitor, while metalloprotease inhibitors such as, 1,10-Phenanthroline and EDTA did not inhibit the proteolytic activity (Figure 2a & b). (Table 1) represents the effect of protease inhibitors on the proteolytic activity of AqSEJ. In order to identify the presence of glycoproteins in the extract PAS staining was done, the AqSEJ showed glycoprotein band at 14.3 kDa region on SDS-PAGE (Figure 1d).

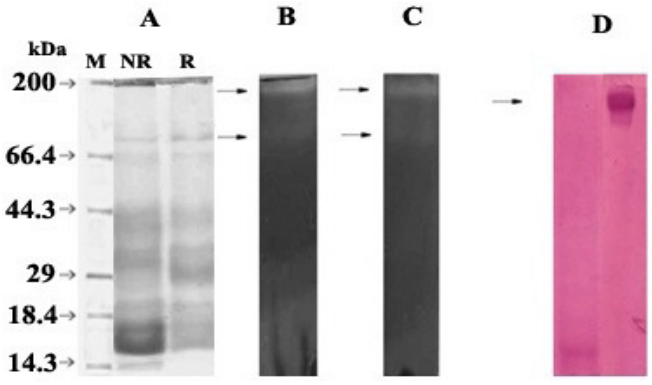

Figure 1: (a) AqSEJ as shown in SDS-PAGE (10 %)

(1) AqSEJ (100 µg) under non-reduced and (2) reduced conditions; (b) Caseinolytic activity staining (zymography) of AqSEJ (60 µg) resolved by SDS-PAGE (10 %) under non-reduced condition; (c) Gelatinolytic activity staining (zymography) of AqSEJ (60 µg) resolved by SDS-PAGE (10 %) under non-reduced condition; (d) PAS staining of AqSEJ (1) and PAS staining of positive control fibrinogen (2). M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), β lactalbumin (18.4) and lysozyme (14.3).

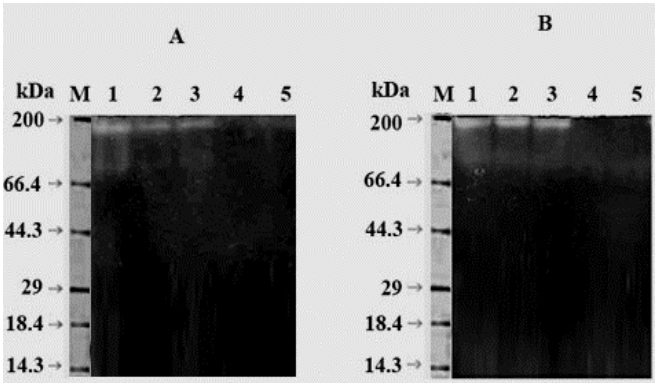

Figure 2: (a) Casienolytic activity of AqSEJ resolved in SDS-PAGE (10 %)

(1) AqSEJ 60 µg alone,(2) AqSEJ 60 µg was pretreated with 5 mM EDTA,(3) AqSEJ 60 µg was pretreated with 5 mM 1,10-Phenanthroline,(4) AqSEJ 60 µg was pretreated with 5 mM PMSF, (5) AqSEJ 60 µg was pretreated with 5 mM IAA under non-reduced condition; (b) Gelatinolytic activity of AqSEJ resolved in SDS-PAGE (10 %), (1) AqSEJ 60 µg alone,(2) AqSEJ 60 µg was pretreated with 5 mM EDTA,(3) AqSEJ 60 µg was pretreated with 5 mM 1,10-Phenanthroline,(4) AqSEJ 60 µg was pretreated with 5 mM PMSF,(5) AqSEJ 60 µg was pretreated with 5 mM IAA under non-reduced condition. M represents the molecular weight marker in kDa from top to bottom: myosin-H-chain (200), BSA (66.4), ovalbumin (44.3), carbonic anhydrase (29), β lactalbumin (18.4) and lysozyme (14.3).

Table 1: Effect of Inhibitors on the Proteolytic Activity of AqSEJ

Inhibitor (5 mM each)	Activity/residual activity
None	100
EDTA	98.95
EGTA	90.20
1,10-Phenanthroline	74.50
IAA	15.40
PMSF	01.80

Values are average of three independent experiments

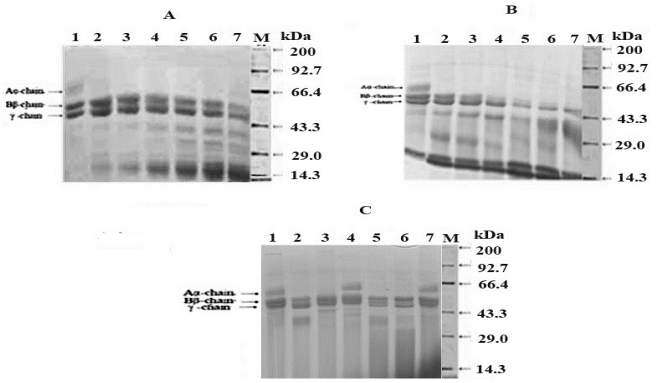

Figure 3: Fibrinogenolytic activity

(a) Dose dependent effect: Fibrinogen alone (1) and fibrinogen treated with 2 µg (2), 4 µg (3), 8 µg (4), 12 µg (5)16 µg (6) and 20 µg (7) of AqSEJ; **(b) Time dependent effect:** AqSEJ (16 µg) was incubated with fibrinogen (50 µg) at 37°C for 0 hour (1), 2 hours (2), 4 hours (3), 8 hours (4), 14 hours (5),18 hours (6) and 24 hours (7); **(c) Inhibition of fibrinogenolytic activity:** AqSEJ (16 µg) was preincubated with and without protease inhibitors for 30 min at 37°C.

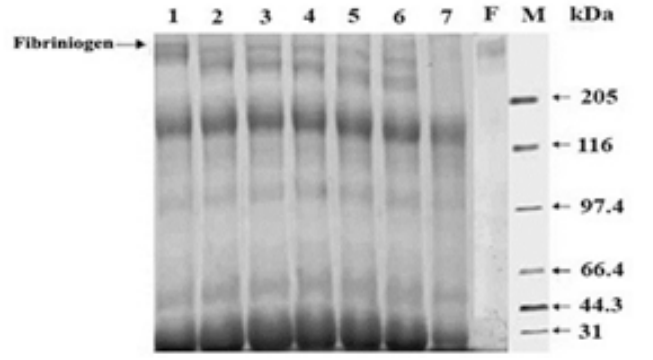

Figure 5: Degradation of plasma proteins:

Plasma protein (100 µg) is incubated with AqSEJ in 40 µL of 10 mM Tris-HCl buffer pH 7.4 at 37°C and then analyzed on 7.5% SDS-PAGE under non-reduced condition. Plasma protein (100 µg) alone (1), plasma protein treated with 5 µg (2), 10 µg (3), 20 µg (4), 30 µg (5), 40 µg (6), 50 µg (7) of AqSEJ of for 12 hours, (F) 20 µg of fibrinogen (positive control).

AqSEJ specifically hydrolyzed fibrinogen and fibrin but not other plasma proteins

AqSEJ hydrolyzed human fibrinogen and fibrin clot. AqSEJ at the concentration of 0-20 µg for the incubation period of 4 hours preferentially degraded Aα chain followed by Bβ chain of fibrinogen in a dose dependent manner (Figure 3a). However, when the incubation period was extended up to 24 hours it hydrolyzed Aα, Bβ and γ chains of fibrinogen (Figure 3b). In both the cases (4 hours and 24 hours) the activity of AqSEJ was authenticated by the progressive decreased intensity of the susceptible fibrinogen chains and appearance of new low molecular weight peptides as degradation products on SDS-PAGE under reduced condition. The fibrinogenolytic activity was completely neutralized by PMSF and IAA (Figure 3c). Furthermore, AqSEJ hydrolyzed washed plasma/fibrin clot and the specific activity was found to be 2.62 units/mg/

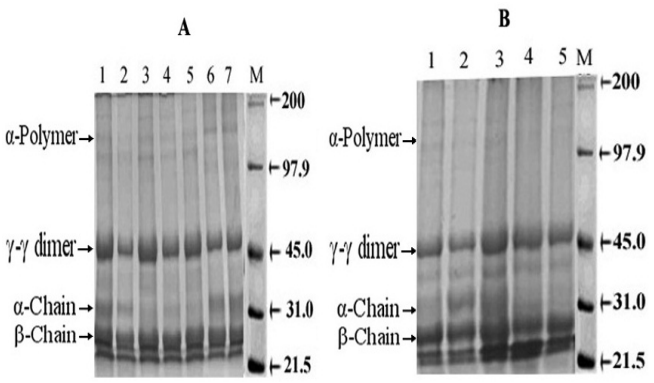

Figure 4: Fibrinolytic activity

(a) Dose dependent effect: plasma clot alone (1) and plasma clot treated with 5 µg (2), 10 µg (3), 20 µg (4), 30 µg (5) of AqSEJ respectively, 30 µg of AqSEJ was preincubated with 5 mM PMSF for 30 min at 37°C (6) and 30 µg of AqSEJ was preincubated with 5 mM IAA (7) for 8 hours at 37°C; **(b) Time dependent effect:** AqSEJ (30 µg) was incubated with plasma clot for 0 hour (1), 2 hours (2), 4 hours (3), 8 hours (4), 12 hours (5).

min. The plasma/fibrin clot hydrolyzed pattern of AqSEJ was also analyzed on the SDS-PAGE. The (Figure 4a & b) represents both dose and time dependent fibrinolytic activity of AqSEJ. AqSEJ preferentially degraded α polymer and α chain of partially cross linked human fibrin clot, while γ-γ dimer and β chain remained resistant to proteolysis over the incubation period of 12 hours. These results suggested that AqSEJ preferentially cleaves the clot and might be used as clot dissolving factor. Since AqSEJ exhibits proteolytic activity and degrades fibrinogen, experiments were carried out to rule out the possibility that AqSEJ exerts its proteolytic activity on other plasma protein. AqSEJ did not hydrolyze any of the plasma protein other than fibrinogen (Figure 5). The intensity of the fibrinogen band was progressively vanished with increase in concentration of AqSEJ (0-50 µg) which is compared with positive control fibrinogen alone.

AqSEJ is devoid of plasminogen activation ability

AqSEJ degraded azocasien but did not activate plasminogen, while streptokinase a positive control activated the plasminogen. These results suggest that AqSEJ specifically acts on fibrinogen and directly dissolves fibrin clot without activating plasminogen to plasmin.

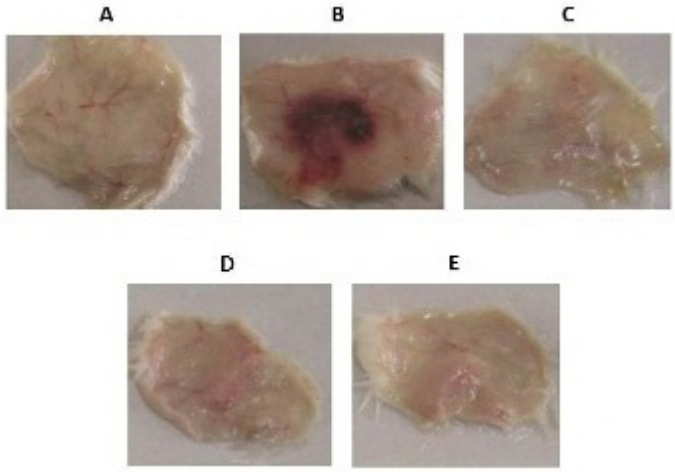

Figure 6: Dose dependent hemorrhagic activity of AqSEJ.

A: saline, B: positive control 2 MDH venom, C: 50 µg, D: 100 µg and E: 200 µg of AqSEJ was injected independently in to mice in a total volume of 50 µL intradermally..

AqSEJ do not cause hemolysis, edema and hemorrhage

AqSEJ did not hydrolyze RBC. Moreover, it did not cause hemorrhage and edema in experimental mice up to the concentration of 200 µg (Figure 6) while positive control Daboia russelli venom induced hemorrhage and edema in experimental mice. These results suggest that the AqSEJ are relatively non-toxic at the tested dose.

DISCUSSION

In recent time, the therapeutic usage of proteolytic enzymes from ticks, earth worm, leach, venom of snake, spider, bee, scorpion and latex of plants have been gaining much importance.[24,27-29] Despite anticancer, antihypertensive, antiulcer, antioxidant, antifungal and antimicrobial activities of Jackfruit,[9,11] its potential role of fibrino (geno) lysis was not explored. Thus, the current study demonstrates the fibrinogen and fibrin clot hydrolyzing activities of AqSEJ.

AqSEJ showed dissimilar protein banding pattern under reducing and non-reducing conditions, suggesting the presence of oligomeric proteins. AqSEJ was positive to PAS staining only at the low molecular weight region, suggesting proteases were devoid of carbohydrate moities. AqSEJ hydrolyzed casein, gelatin, fibrinogen and fibrin suggesting proteolytic activity. Proteolytic activity was also confirmed using casein and gelatin zymography, AqSEJ exhibited two translucent activity bands of higher molecular weight region, suggests the presence of two isoforms of proteases. Interestingly, proteolytic activity of the AqSEJ was completely neutralized only by PMSF and IAA indicating the presence of serine and cystein proteases respectively.

Fibrinogen (factor I) is a large plasma glycoprotein having the molecular mass of 340 kDa, comprises of three polypeptide chains Aα, Bβ and γ, play an important role in fibrin clot formation. Physiologically thrombin specifically degrades Aα and Bβ chains of fibrinogen from N-terminal end and generates fibrinopeptide A and B that facilitates the generation of fibrin.[27,30] The proteolytic enzymes may degrade fibringogen either from N-terminal end or C terminal end. The proteolytic enzymes (serine, metallo and cystein) degrade fibrinogen similar to thrombin and are considered to be thrombin like enzymes.[30] *Artocarpus heterophyllus* latex completely degraded α chain, while partially degraded β and γ chains of human fibrinogen.[15] AqSEJ non-specifically hydrolyzed all the chains (Aα, Bβ andγ) of fibrinogen; therefore it is too premature to say the site of action of proteolytic enzyme on the fibrinogen.

Fibrinolysis is an important event in preventing blood clot, wound healing and normal circulation of the blood. Physiologically plasmin hydrolyzes the fibrin.[27-29] Interestingly, AqSEJ preferentially hydrolyzed α polymer and α chain of human fibrin, thus it may serve as a clot dissolving factor in thrombotic disorder. It is to emphasis that, AqSEJ degraded all the chains of fibrinogen, α polymer and α chain of human fibrin but did not hydrolyze other human plasma proteins revealed its specificity towards only fibrinogen and fibrin. The fibrino(geno)lytic activity was completely abolished by PMSF, IAA but did not EDTA and 1,10-Phenanthroline revealed the role of serine and cysteine proteases in the fibrino(geno)lytris. Moreover, AqSEJ did not activate plasminogen to plasmin revealed its direct action on fibrin.

Interestingly, AqSEJ was non-toxic to experimental mice as it did not show indirect hemolytic activity, hemorrhage and edema in experimental mice.

CONCLUSION

This study reports on the fibrino(geno)lytic activities of AqSEJ. Hence, further identification and biochemical characterization of active fibrino(geno)lytic enzyme from the AqSEJ may present a promising alternative in the treatment of thrombotic disorders.

CONFLICT OF INTEREST

No competing financial interest exists.

ACKNOWLEDGMENTS

The authors thank the Vision Group on Science and Technology, Government of Karnataka, Bangalore and UGC New Delhi for financial assistance.

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Flavonoids from *Eupatorium illitum* and Their Antiproliferative Activities

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ABSTRACT

Objective: To isolate the chemical constituents of the aerial parts from *Eupatorium illitum*, elucidate their structures and evaluate their antiproliferative activity on human cancer cell lines. **Materials and Methods:** The ethanolic extract of *Eupatorium illitum* afforded five compounds, which were characterized using spectroscopic techniques and by comparison with data from the literature. Antiproliferative activities of selected isolates were evaluated. **Results:** The flavonoids Kumatakenin (**1**), Ermanin (**2**), 7-methoxy-aromadendrin (**3**) and Naringenin (**4**), together with 4-hydroxybenzoic acid (**5**) were isolated. Compounds **1**, **2**, **4** and **5** were evaluated for their antiproliferative activity on the human cancer cell lines A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), and T-47D (breast) presenting a wide range of bioactivities. In general, best results were observed for **5**. **Conclusion:** Compounds **1-5** are reported for first time from *Eupatorium illitum*. Isolated phytochemicals show moderate to low antiproliferative activities when evaluated on the aforementioned human cancer cell lines.

Key words: *Eupatorium illitum*, Ermanin, 4-Hydroxybenzoic acid, Kumatakenin, 7-Methoxy-aromadendrin, Naringenin.

INTRODUCTION

Eupatorium illitum Urb. (Asteraceae) is an endemic plant from Dominican Republic.[1] The genus Eupatorium comprises of nearly 1,200 species distributed mainly in the tropical regions of Americas, Europe, Africa and Asia[2]. Flavonoids isolated from species of this genus have been reported to show cytotoxic activity against cancer cell lines.[3-5] In the present study, we report compounds **1-5**, isolated for first time from *E. illitum*, and their antiproliferative activities on human cancer cell lines A549, HBL-100, SW1573, HeLa, and T-47D.

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DOI: *10.5530/pj.2015.3.6*

MATERIALS AND METHODS

General

IR spectra were recorded using a Hyperion 3000 Fourier transform infrared microscope. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance III spectrometer with cryoprobe operating at 700 MHz in ^{1}H and 175 MHz in

^{13}C NMR respectively. DMSO–d_6 was used as solvent. HRESIMS was performed with a Bruker Maxis 4G QTOF mass spectrometer; presented results are for negative ion mode. Silica gel 60 (particle sizes 0.040-0.063 mm and 0.015-0.040 mm, Merck KGaA, Darmstadt, Germany) was used for column chromatography. Analytical and preparative TLC was developed on Silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany).

Plant Material

The aerial parts of *Eupatorium illitum* Urb. (Asteraceae) were collected on June 2010 at Sierra de Bahoruco, Provincia Pedernales, Dominican Republic. The plant material was identified by Teodoro Clase, botanist at Jardín Botánico Nacional "Dr. Rafael Ma. Moscoso", Santo Domingo, Dominican Republic, where a voucher specimen (JBSD 121457) has been deposited.

Extraction and Isolation

Aerial parts of *Eupatorium illitum* were shade, air dried, and ground to a fine powder. The ground material (617 g) was exhaustively extracted with 95% EtOH in a Soxhlet apparatus. The resulting crude extract (90.9 g) was dissolved in 95% EtOH (1.2 L) and treated with a 5% lead acetate solution (1L) to precipitate chlorophyll. After 24 hours, the mixture was filtered over paper, concentrated *in vacuo* to remove most of the EtOH, and extracted successively with hexane (6 x 750 mL) and ethyl acetate (6 x 1L). The ethyl acetate residue (10.7 g) was washed with distilled water (2 x 1L), dried over anh. Na_2SO_4, and subjected to CC (Si gel 0.040-0.063 mm) using hexane-acetone mixtures of increasing polarity to afford 22 fractions (F1-F22). Fraction 15 afforded **1** (190.6 mg) as a yellow precipitate; supernatant F15 was eluted over a column of Si gel (0.015-0.040 mm) with a gradient system of hexane-AcOEt (3:1 to pure AcOEt) affording 19 subfractions. Subfraction F15-13 gave **2** (9.2 mg) after PTLC (Si gel) using a mixture of hexane-AcOEt (7:3).

Fraction 16 was chromatographed over a column of Si gel (0.015-0.040 mm) with a gradient system of hexane-AcOEt (7:3 to pure AcOEt), affording 16 subfractions. Sub fraction F16-15 was recromatographed over Si gel (0.015-0.040 mm) using a gradient system of hexane-AcOEt (7:3 to pure AcOEt) to yield 21 subfractions. PTLC (Si gel) of subfraction F16-15-15 using a mixture of hexane-AcOEt (6.5:3.5, twice) afforded **3** (5.8 mg) and **4** (5.6 mg).

Fraction 21 was chromatographed over a Si gel (0.015-0.040 mm) column with a gradient system of hexane-acetone (6.5:3.5 to pure acetone) to yield **5** (13.9 mg).

Antiproliferative assays

Biology

All starting materials were commercially available research-grade chemicals and used without further purification. Fetal calf serum (FCS) was purchased from Gibco (Grand Island, NY), trichloroacetic acid (TCA) and glutamine were from Merck (Darmstadt, Germany), and RPMI 1640 medium penicillin G, streptomycin, DMSO and sulforhodamine B (SRB) were from Sigma (St Louis, MO).

Cell lines and culture

The human solid tumor cell lines A549, HBL-100, HeLa, SW1573, and T-47D were used in this study. These cell lines were a kind gift from Prof. G. J. Peters (VU Medical Center, Amsterdam, The Netherlands). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum and 2 mM L-glutamine in a 37ºC, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and re-suspended in antibiotic containing medium (100 units penicillin G and 0.1 mg of streptomycin per mL). Single cell suspensions were counted using Orflow's Moxi^Z automated cell counter (Ketchum, ID) and dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 µL per well at densities of 10 000 (A549, HBL-100, HeLa and SW1573), and 15 000 (T-47D) cells per well, based on their doubling times.

Chemosensitivity testing

Compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 1–100 µM. The drug treatment was started on day 1 after plating. Drug incubation times were 48 h, after which time cells were precipitated with 25 µL ice-cold TCA (50% w/v) and fixed for 60 min at 4ºC. Then the SRB assay was performed.[6] The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader (Winooski, VT). Values were corrected for background OD from wells only containing medium.

RESULTS AND DISCUSSION

The ethyl acetate residue (10.7 g) of the ethanolic extract from *E. illitum*, afforded, after chromatographic

procedures, compounds **1-5** (Figure 1). Their chemical structures were identified using spectroscopic techniques (FTIR, HRESIMS, and NMR) and by comparison with data reported in literature. Their found spectral data are shown below.

Kumatakenin (1)

Yellow powder; IR ν_{max}: 3236, 2948, 2362, 2337, 1656, 1600, 1583, 1497 cm^{-1}; HRESIMS obsd. m/z 313.0663 [M – H]$^-$ calcd. for $C_{17}H_{14}O_6$, 313.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.69 (1H, s, 5-OH), 10.31 (1H, br s, 4'-OH), 7.99 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.97 (2H, d, *J* = 8.6 Hz, H-3', H-5'), 6.76 (1H, d, *J* = 1.6 Hz, H-8), 6.39 (1H, d, *J* = 1.6 Hz, H-6), 3.87 (3H, s, 7-OMe), 3.81 (3H, s, 3-OMe). ^{13}C NMR (176MHz, DMSO-d_6) δ = 178.5 (C-4), 165.6 (C-7), 161.4 (C-5), 160.8 (C-4'), 156.8 (C-9), 156.4 (C-2), 138.3 (C-3), 130.7 (C-2', C-6'), 120.9 (C-1'), 116.1 (C-3', C-5'), 105.7 (C-10), 98.2 (C-6), 92.8 (C-8), 60.2 (3-OMe), 56.6 (7-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[7,8] Assignments were confirmed by HSQC and HMBC experiments.

Ermanin (2)

Yellow solid; IR ν_{max}: 3130, 2936, 2840, 2361, 2050, 1650, 1604, 1498 cm^{-1}; HRESIMS obsd. m/z 313.0706 [M – H]$^-$ calcd. for $C_{17}H_{14}O_6$, 313.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.65 (1H, s, 5-OH), 8.03 (2H, d, *J* = 8.8 Hz, H-2', H-6'), 7.14 (2H, d, *J* = 9.1 Hz, H-3', H-5'), 6.46 (1H, d, *J* = 1.7, H-8), 6.21 (1H, d, *J* = 1.9 H-6), 3.87 (3H, s, 4'-OMe), 3.80 (3H, s, 3-OMe). ^{13}C NMR (176MHz, DMSO-d_6) δ = 178.0 (C-4), 164.4 (C-7), 161.4 (C-4'), 161.3 (C-5), 156.5 (C-9), 155.2 (C-2), 138.0 (C-3), 130.0 (C-2', C-6'), 122.2 (C-1'), 114.3 (C-3', C-5'), 104.2 (C-10), 98.7 (C-6), 93.9 (C-8), 59.8 (3-OMe), 55.5 (4'-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[9,10] Assignments were confirmed by HSQC and HMBC experiments.

7-Methoxy-aromadendrin (3)

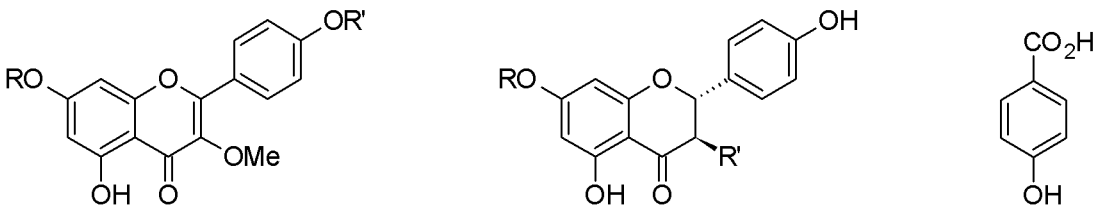
Yellow solid; IR ν_{max}: 3144, 2939, 2258, 1716, 1636, 1616, 1595, 1573, 1501, 1443 cm^{-1}; HRESIMS obsd. m/z 301.0717 [M – H]$^-$ calcd. for $C_{16}H_{14}O_6$, 301.0790; ^{1}H NMR (700MHz, DMSO-d_6) δ = 11.88 (1H, s, 5-OH), 9.57 (1H, s, 4'-OH), 7.33 (2H, d, *J* = 8.2 Hz, H-2', H-6'), 6.80 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.12 (1H, d, *J* = 2.1 Hz, H-6), 6.10 (1H, d, *J* = 1.9 Hz, H-8), 5.82 (1H, d, *J* = 6.4 Hz, 3-OH), 5.11 (1H, d, *J* = 11.3, H-2), 4.66 (1H, dd, *J* = 11.5, 6.3 Hz, H-3), 3.79 (3H, s, 7-OMe); ^{13}C NMR (176MHz, DMSO-d_6) δ = 199.0 (C-4), 168.0 (C-7), 163.4 (C-5), 163.0 (C-9), 158.2 (C-4'), 130.0 (C-2', C-6'), 127.9 (C-1'), 115.4 (C-3', C-5'), 101.8 (C-10), 95.4 (C-6), 94.3 (C-8), 83.5 (C-2), 72.0 (C-3), 56.4 (7-OMe). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[11] Assignments were confirmed by HSQC and HMBC experiments.

Naringenin (4)

Yellow solid; IR ν_{max}: 3115, 3055, 2019, 2819, 2831, 2700, 2349, 2286, 2051, 1898, 1627, 1600, 1586, 1519, 1496 cm^{-1}; HRESIMS obsd. m/z 271.0603 [M – H]$^-$ calcd. for $C_{15}H_{12}O_5$, 271.0685; ^{1}H NMR (700MHz, DMSO-d_6) δ = 12.16 (1H, s, 5-OH), 10.80 (1H, br s, 7-OH), 9.59 (1H, br s, 4'-OH), 7.32 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.80 (2H, d, *J* = 8.3 Hz, H-3', H-5'), 5.88 (2H, s, H-6, H-8), 5.45 (1H, dd, *J* = 12.8, 2.8 Hz, H-2), 3.28 (1H, dd, *J* = 17.0, 12.9, H-3 trans.), 2.69 (1H, dd, *J* = 17.1, 2.9 Hz, H-3 cis.); ^{13}C NMR (176MHz, DMSO-d_6) δ = 196.8 (C-4), 167.2 (C-7), 163.9 (C-5), 163.4 (C-9), 158.2 (C-4'), 129.3 (C-1'), 128.8 (C-2', C-6'), 115.6 (C-3', C-5'), 102.2 (C-10), 96.3 (C-6), 95.5 (C-8), 78.9 (C-2), 42.4 (C-3). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[12,13]

4-Hydroxybenzoic acid (5)

White solid; IR ν_{max}: 3400, 2255, 2128, 1654, 1049, 1023, 999, 824, 762, 625, 614 cm^{-1}; HRESIMS obsd. m/z 137.0242 [M – H]$^-$ calcd. for $C_7H_6O_3$, 137.0317; ^{1}H NMR (700MHz,



1 R = CH_3, R' = H

2 R = H, R' = CH_3

3 R = CH_3, R' = OH

4 R = H, R' = H

5

Figure 1: Structures of compounds 1-5 isolated from the aerial parts of *Eupatorium illitum*

Table 1: Values of GI_{50} (μg.ml^{-1}) for selected compounds from *Eupatorium illitum* on human cancer cell lines

Compound	A549	HBL-100	SW1573	HeLa	T-47D
1	163	12	5	10	250
2	16	11	7	8	58
4	40	22	10	7	49
5	12	10	8	7	13

DMSO-d_6) δ = 7.79 (2H, d, *J* = 8.5 Hz, H-2, H-6), 6.82 (2H, d, *J* = 8.7 Hz, H-3, H-5); ^{13}C NMR (176MHz, DMSO-d_6) δ = 167.6 (COOH), 162.0 (C-4), 132.0 (C-2, C-6), 121.8 (C-1), 115.6 (C-3, C-5). The spectroscopic data (^{1}H and ^{13}C NMR) were comparable with published values.[14]

Antiproliferative activity

Selected isolates were evaluated for their antiproliferative activity against the human solid tumor cell lines A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), and T-47D (breast). The effect of these compounds on the aforementioned cell lines, expressed as the value GI_{50} (concentration causing 50% of growth inhibition), is presented in Table 1.

CONCLUSION

In summary, we have reported the isolation of four flavonoids from Eupatorium illitum in addition to 4-Hydroxybenzoic acid. All compounds are reported for first time in this plant. The study of the antiproliferative activity against human solid tumor cell lines showed that the compounds are able to inhibit cell growth in all cell lines tested.

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

ACKNOWLEDGMENTS

This project has been supported by research grant **FONDOCYT 2009-16**, Ministerio de Educación Superior, Ciencia y Tecnología (MESCYT), Dominican Republic. QAC thanks Dr. Mehdi Keramane (Mc Master University, Canada) for a diligent technical assistance. JMP thanks the EU Research Potential (FP7-REGPOT-2012-CT2012-31637-IMBRAIN), the European Regional Development Fund (FEDER), and the Spanish Instituto de Salud Carlos III (PI11/00840) for financial support. GBP thanks Fundación Caja Canarias for a postgraduate grant.

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Antioxidant and DNA Damage Protective Effects of *Asparagus racemosus* in Human Colon and Mice Muscle Cells

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ABSTRACT

Introduction: The present study was designed to investigate the *in vitro* antioxidant and macromolecule damage protective effects of *Asparagus racemosus* water (AWE) and methanolic (AME) fractions of roots. **Methods:** The *in vitro* antioxidant activity of AWE/AME was estimated by free radical scavenging assays. The DNA damage of HT29 and C2C12 cells was analyzed by comet assay. The plasmid DNA damage and protein oxidation were carried out by agarose gel electrophoresis and SDS-PAGE analysis respectively, where as lipid peroxidation was performed by TBARS assay. **Results:** Both the extracts showed scavenging activity with IC_{50} values of 417.4 ± 19.5 / 298 ± 13.5, 381 ± 18.2 / 235 ± 11.9, 54.8 ± 2.95 / 31.6 ± 1.52, 28.9 ± 1.73 / 19.7 ± 1.55 µg/mL for DPPH, metal chelating, ABTS and Nitric oxide scavenging activities respectively. Similarly the methanolic extract showed more potent reducing power and total antioxidant activities over water fraction. The AME showed 56.8% and 41.2% protection against H_2O_2 (Hydrogen peroxide) induced DNA damage of HT29 human colon cells and C2C12 murine myoblasts. The extract also showed protection against H_2O_2 induced plasmid DNA damge, AAPH induced protein oxidation of bovine serum albumin (BSA) and lipid peroxidation of rat hepatic tissue. **Conclusion:** Over all this study showed remarkable antioxidant and macromolecule damage protective effects of *A.racemosus*. The observed biological properties may be attributed to the high content phenols and flavonoids in the methanolic extract *A. racemosus* over water extract.

Key words: AAPH, *Asparagus racemosus*, C2C12, HT29, Protein oxidation, Single cell gel electrophoresis.

INTRODUCTION

The imbalance between oxidants and antioxidants leading to damage of the tissues is known as oxidative stress. The reactive species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitrogen oxide (NO^-), peroxynitrite ($ONOO^-$) and hypochlorous acid (HClO) are all the common products of regular metabolism. However under stress conditions the excessive production of these reactive species exerts harmful effects. On the other hand the antioxidants are known to terminate chain reactions initiated by free radicals.[1]

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DOI: *10.5530/pj.2015.3.7*

Asparagus racemosus known as shatavari belongs to family Liliaceae and is a climbing plant which grows across India. The metabolites of the herb are racemosol, isoflavones, sarsasapogenin glycoside, steroidal sapogenins, ecdysteroids, asparinins, asparosides, curillins, curillosides, shavatarins, fructo-oligosaccharides and fatty acids.[2-5] In the traditional system of medicine the herb is used as demulscent, diuretic, aphrodisiac and galactogogue.[6] *A. racemosus* is also used to prevent ageing, to increase longevity, to improve mental function and to boost immunity.[7,8] Further reports indicate the pharmacological activities of *A. racemosus* root that include antiulcer and antidiabetic activities.[9,10]

In the present investigation we aimed to study the free radical induced *in vitro* antioxidant scavenging activity and macromolecule damage protective effects of *A. racemosus* aqueous and methanolic extract against H_2O_2 induced

DNA damage of human colon and mice muscle cells, AAPH induced protein and hepatic tissue damage.

MATERIALS AND METHODS

Chemicals and reagents

Hydrogenperoxide was procured from Merck (Bangalore, India), gallic acid, catechin, AAPH, BSA, DMEM, fetal bovine serum, penicillin, streptomycin solution from Sigma (Bangalore, India), horse serum from Hyclone (Bangalore, India), whereas other chemicals used were of analytical grade and procured from Hi-Media (Bangalore, India).

Preparation of methanolic extract

The methanolic extract of *Asparagus racemosus* (AME) was prepared as described earlier.[11] Briefly the roots were washed, cut into small pieces, shade dried and powdered. The powdered material was extracted using methanol and concentrated under reduced pressure using rotary flash evaporator (Rotavac, Schwabach, Germany). Whereas the aqueous extract (AWE) was prepared by maceration of *A. racemosus* root powder with water, followed by filtration and lyophilization (Lyolab, Hyderabad, India).

Estimation of total polyphenol content

Total polyphenol content was determined using Folin–Ciocalteu reagents where as gallic acid was used as a standard. Gallic acid 20-100 µg was added to the test tubes and the volume was made up to 3 mL using distilled water. *A. racemosus* extracts (1 mg/mL) were taken in the range of 100-1000 µg, and the volume was made up to 3 mL using distilled water and 0.5 mL of Folin-Ciocalteu reagent was added to all the tubes followed by incubation for 6 mins. To this mixture 2 mL of 7% sodium carbonate was added and the absorbance was measured at 650 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). The results were expressed in terms of µg (gallic acid equivalents) GAE/mg of extract.

Determination of flavonoid content

The plant extracts/catechin as standard (1mg/mL) were added at different concentrations and made to one mL with distilled water. To this 75 µL of 5% $NaNO_2$ and 150 µL of 10% $AlCl_3$ were added and incubated for a period of 10 mins. To this mixture 500 µL of 1M NaOH and 775 µL of distill water was added and the reaction was measured at 510 nm against reagent blank. The results were expressed in terms of µg (catechin equivalents) CE/mg of extract.

In vitro antioxidant scavenging activities

Total antioxidant activity

The total antioxidant activity of AWE and AME were evaluated spectrophotometrically. The plant extracts 1 mg/mL or gallic acid as standard (1 mg/mL) were added to the tubes and finally the volume was made to 0.3–mL using distilled water followed by 3 mL of reagent mixture (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 mins. After cooling to room temperature, the absorbance of each sample was measured at 695 nm against the blank and the results were expressed in terms of µg GAE/mg of extract.

Reducing power

The total reducing power was evaluated by using ascorbic acid as standard (1mg/mL) and plant extracts (1 mg/mL) added in different dilutions to the test tubes and the volume was made up to 1 mL using distilled water. To this 2.5 mL of PBS and 1% potassium ferric cyanide were added and the reaction mixtures were incubate at 50°C for 20 mins. Finally to the tubes 2.5 mL of 10% TCA and 0.1% of 0.5 mL ferric chloride and 2.5 mL of distilled water were added. The absorbance of each sample was measured at 700 nm using a spectrophotometer and the results were expressed in terms of µg (ascorbic acid equivalents) AAE/mg of extract.

DPPH radical Scavenging assay

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) is a stable free radial with violet color and the scavenging of DPPH radicals generate yellow color. Based on this principle herbal extracts/bioactive compound have been checked for their free radical scavenging activity. The water/methanolic extracts and BHA (1 mg/mL) as standard were added and the volume is made up to 3 mL using methanol. To this freshly prepared DPPH solution was added and the tubes were incubated at room temperature for 45 mins. The absorbance was read at 515 nm and the results were expressed in terms of IC_{50} values.

Metal chelating activity

The metal chelating activity was determined using ferrozine reagent with EDTA as standard. The extracts (1 mg/mL) and EDTA (0.1 mg/mL) were taken in different concentrations in the test tubes and the volume was made up to 3 mL using distilled water. To this 50 µL of ferric chloride and 2 mL of ferrozine were added and incubated for 10 mins. at room

temperature. The absorbance was measured at 562 nm and the results were expressed in terms of IC_{50} values.

ABTS radical scavenging activity

The ABTS radical scavenging activity was evaluated using ascorbic acid as standard. Prior to the assay the ABTS reagent was prepared by mixing 7 mM ABTS and 2.45 mM potassium per sulphate and the solution was diluted with ethanol to get a blue-green chromophore with an absorbance of 0.700 ± 0.025 at 734 nm. The extracts (1 mg/mL) were added in different concentrations and the volume was made up to 1 mL using distilled water. To this 1 mL of ABTS reagent was added and the samples were incubated at room temperature for 6 mins. The absorbance of each sample was measured at 734 nm and the results were expressed in terms of IC_{50} values.

Nitric oxide scavenging activity

This assay was performed as described by Sreejayan and Rao (1997).[12] Brifely, sodium nitroprusside (10 mM) in PBS was mixed with different concentrations of extracts/standard and incubated at room temperature for 150 mins. The reaction mixture without the sample/standard served as the control. After incubation, 0.5 mL of Griess reagent [1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1- naphthyl) ethylenediamine HCl] was added and the absorbance was recorded at 546 nm. L-ascorbic acid was used as a reference standard and the results were expressed in terms of IC_{50} values.

Hydroxyl radical scavenging activity

This assay was performed according to the method of Kunchandy and Rao (1990).[13] The Fenton reaction was initiated using Fe^{3+}-ascorbate-EDTA-H_2O_2 to generate hydroxyl radicals which degrades DNA deoxyribose. The reaction mixture composed of 2-deoxy-2-ribose (2.8 mM); KH_2PO_4–KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and different concentrations of (0-200 µg/mL) of extracts/standard (ascorbic acid). After incubation for 1 h at 37°C, 0.5 mL of the reaction mixture was added to 1 mL of 2.8% trichloroacetic acid. Finally 1 mL of 1% TBA was added and the mixture was incubated at 90°C for 15 mins. The developed color product was measured at 532 nm and the results were expressed in terms of IC_{50} values.

Macromolecule damage protective activity

Plasmid DNA damage nick assay

The protective effect of *A. racemosus* methanolic extract against H_2O_2 induced DNA damage was studied using pRSET-A plasmid DNA as described by Russo et al. (2000).[14] Plasmid DNA (350 ng) in 10 µL of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), H_2O_2 was added at a final concentration of 10 mM with or without ARE (2.5, 5 and 10 µg/mL)/gallic acid (5 µg/mL) and incubated for a period of 5 mins at room temperature. Plasmid DNA without any treatment was used as control. After due incubations, the plasmid DNA was electrophoresed on 1% agarose gel. Finally the gel was stained with ethidium bromide and photographed using gel documentation system (Syngene, Cambridge, UK).

Cell culture

HT29 Human colon carcinoma cells and C2C12 murine myoblasts were procured from National Centre for Cell Sciences, Pune, India.

HT29 Human colon carcinoma cells

HT29 cells (4 X 10^5 cells/mL) were grown in 75 cm^2 flasks supplemented with Dulbecco's modified eagle's medium (DMEM) and 10% fetal bovine serum, containing, penicillin and streptomycin solution in humidified atmosphere at 37 ºC with 5% CO_2.

C2C12 murine muscle cells

C2C12 murine muscle cells (4 X 10^5 cells/mL) were grown in 75 cm^2 flasks supplemented with 1:1 mixture of DMEM and 10% fetal bovine serum, containing, penicillin and streptomycin solution in humidified atmosphere at 37 ºC with 5% CO_2 for 5 days. The differentiation of muscle cells to myoblasts was carried out by changing the cells to DMEM with 2 % horse serum.

Single cell gel electrophoresis

Once the confluency reached HT29 and C2C12 cells were collected by trypsinization and washed twice with PBS to remove serum and media. The cells were treated with 200 µM H_2O_2 with or without pretreatment of *A. racemosus* (100 µg/assay). The DNA damage and the protective effect of AME were measured by alkaline comet assay as described.[15] The cells were mixed with 100 µL of 0.7% (w/v) low melting agarose (LMA) and pipetted on to the frosted slides pre-coated with 1.0% (w/v) normal melting agarose. After solidification, the slides were covered with another 100 µL of 0.7% (w/v) LMA and immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, 0.1% SDS and 1% Triton X-100 and 10% DMSO;

pH 10.0) for 90 mins. After lysis the slides were transferred to electrophoresis tank with unwinding buffer (3 M NaOH, 10 mM EDTA; pH 13.0) for DNA unwinding. Later the slides were run with an electric current of 25 V/300 mA for 20 mins. Followed by unwinding the slides were washed twice with neutralizing buffer (0.4 M Tris– HCl; pH 7.5). Finally the DNA in the agarose gels was stained with ethidium bromide (20 µg/mL) and the DNA damage was observed and photographed using fluorescence microscope (Olympus, Japan) equipped with Cool SNAP® Pro color digital camera. The tail length and per cent inhibition of DNA damage with AME treatment was determined using Image Pro® plus software.

Protein oxidation

The oxidation of bovine serum albumin (BSA) was carried using AAPH, which is an initiator of peroxyl radicals upon decomposition with water.[16] BSA (5 µg) was dissolved in phosphate buffer (pH 7.3) and incubated for 2 h with or without 40 mM AAPH and in the presence or absence of AME. Finally the protein samples were subjected to SDS-PAGE electrophoresis. The gels were stained with 0.15% coomassie brilliant blue R-250 and the amount of protein damage was quantified by measuring the density of each band using NIH Image J software.

Lipid peroxidation

The lipid peroxidation was spectrophotometrically estimated in terms of thiobarbituric acid reactive substances (TBARS) formed in the liver homogenate treated with AAPH radicals as described by Wright et al. (1981).[17] Liver tissues were collected from 3-4 months old male Wistar albino rats weighing 120 ± 12.5 g and washed with 0.95% NaCl solution. Liver homogenates were prepared in ice-cold 3 mM tris buffer containing 250 mM sucrose and 0.1 mM EDTA (pH 7.4). To 0.5 mL of liver homogenates 0-100 µL of AME was added and made up to 1 mL with phosphate buffer (0.1 M, pH 7.4). Lipid peroxidation was initiated by treatment with 1 mL of 200 µM AAPH followed by incubation at 37°C for 2 h and the reaction was terminated by addition of 1.0 mL of TCA (10%, w/v). Finally 1.0 mL of TBA (0.67%, w/v) was added and the tubes were placed in a boiling water bath for 20 mins. Samples were centrifuged at 2,500 g for 10 mins. and the malondialdehyde formed in each sample was assessed by measuring the absorbance at 535 nm against a reagent blank. BHA was used as a standard antioxidant.

RESULTS

Total polyphenolic and flavonoid content

The water and methanolic fractions of *A. racemosus* showed 146 ± 8.2 and 246 ± 11.8 µg GAE/mg total polyphenols wher as the flavonoid content was found to be 113 ± 6.8 and 154 ± 7.4 µg CE/mg respectively. These results shows that methanolic fraction possess high content of polyphenols and flavonoids over water fraction of *A. racemosus* (Table 1).

In vitro antioxidant and free radical scavenging activity

The water and methanolic fractions of *A. racemosus* were further analysed for their antioxidant activities by an array of *in vitro* antioxidant assays. Our results demonstrates that AWE/AME exhibits total antioxidant and reducing power activities of 229 ± 12.3/349 ± 17.7 µg GAE/mg and 162 ± 9.3/230 ± 10.3 µg AAE/mg respectively. Whereas the DPPH, metal chelating, ABTS, NO and hydroxyl radical scavenging activities of AWE/AME falls in the range of

Table 1: Demonstrate that methanolic extract of *A. racemosus* inhibits the free radicals more effectively compared with the water fraction.

Assay	Water extract (AWE)	Methanolic extract (AME)
Total polyphenolic content	146 ± 8.2 µg GAE/mg	246 ± 11.8 µg GAE/mg
Total flavonoids	113 ± 6.8 µg CE/mg	154 ± 7.4 µg CE/mg
Total antioxidant activity	229 ± 12.3 µg GAE/mg	349 ± 17.7 µg GAE/mg
Reducing power	162 ± 9.3 µg AAE/mg	230 ± 10.3 µg AAE/mg
DPPH	417.4 ± 19.5 IC50 (µg/ml)	298 ± 13.5 IC50 (µg/ml)
Metal chelating	381 ± 18.2 IC50 (µg/ml)	235 ± 11.9 IC50 (µg/ml)
ABTS	54.8±2.95 IC50 (µg/ml)	31.6±1.52 IC50 (µg/ml)
NO scavenging	28.9±1.73 IC50 (µg/ml)	19.7±1.55 IC50 (µg/ml)
Hydroxyl radical scavenging activity	312±16.55 IC50 (µg/ml)	251±12.35 IC50 (µg/ml)
Anti-Lipid peroxidation	514±23.75 IC50 (µg/ml)	437 ± 22.7 IC50 (µg/ml)

IC_{50} values of 417.4 ± 19.5/298 ± 13.5, 381 ± 18.2/235 ± 11.9, 54.8 ± 2.95/31.6 ± 1.52, 28.9 ± 1.73/19.7 ± 1.55, 312 ± 16.55/251 ± 12.35 µg/ml respectively. These results demonstrate that methanolic extract of *A. racemosus* inhibits the free radicals more effectively compared with the water fraction (Table 1).

Macromolecule damage protective effects of *A. racemosus*

Based on the above assays it is confirmed that methaolic fraction of *A. racemosus* is more potent and the same was used to analyze chemicals (H_2O_2/AAPH) induced macromolecues (DNA, protein and lipid) damage protective effects.

Protective effects of AME against H_2O_2 induced Plasmid DNA damage

The methanolic extract was evaluated for its protective effect on H_2O_2 induced damage of pRSET-A plasmid DNA and analyzed by agarose gel electrophoresis (Figure 1). Negative control (lane-1) showed two bands on agarose gel electrophoresis wherein the faster moving band corresponded to the native supercoiled circular DNA (ScDNA) and the slow moving faint band corresponded to the open circular form (OcDNA). Whereas DNA challenged with H_2O_2 (lane 2) resulted in conversion of supercoiled DNA to linear form indicating the hydroxyl

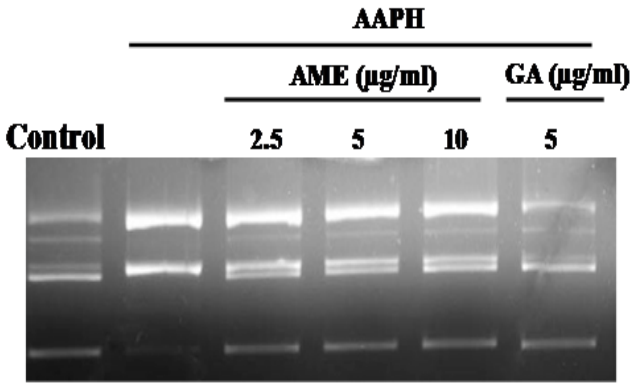

Figure 1: The protective effect of *A. racemosus* on H_2O_2 induced Plasmid DNA damage analysis by agarose gel electrophoresis

radical mediated DNA damage. In the presence of 2.5, 5, 10 µg/mL of *A. racemosus* and 5 µg/mL of gallic acid treatment significant protection to the damage of ScDNA was observed in a dose dependent manner (lanes 3-6).

AME protects H_2O_2 induced colon and muscle cell damage

Further the DNA damage protective effects of *A. racemosus* was verified by single cell gel electrophoresis assay against H_2O_2 induced DNA damage of HT29 human colon and C2C12 murine muscle cells. The DNA damage pattern of HT 29 and C2C12 cells was observed in the form of comets

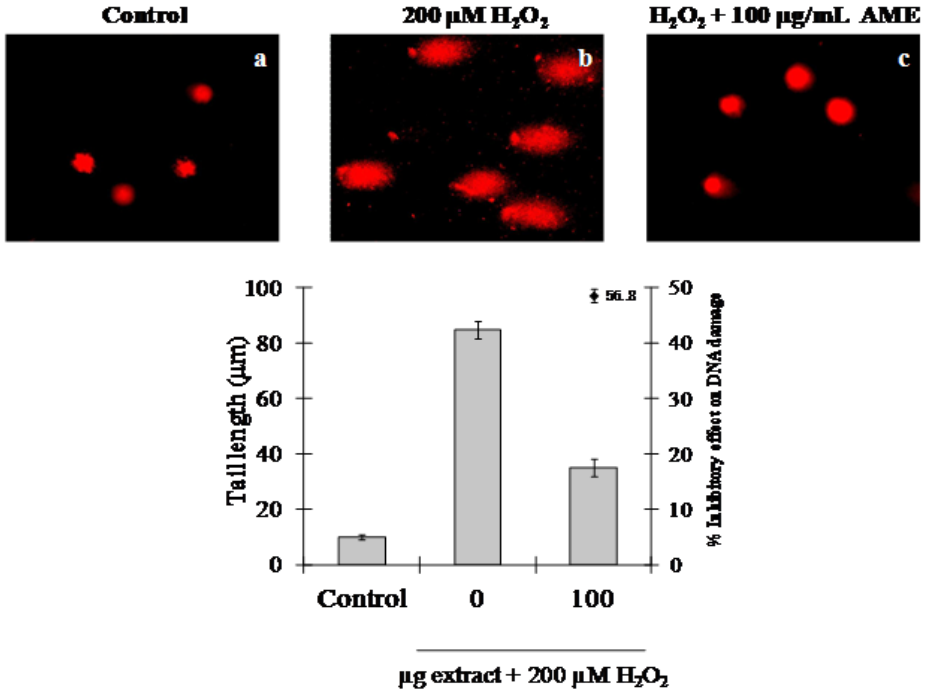

Figure 2 A: The protective effect of *A. racemosus* on H_2O_2 induced DNA damage of HT29 human colon cells by single cell gel electrophoresis assay. A. control cells B. 200 µM H_2O_2 treated cells C. 200 µM H_2O_2 + 100 µg/reaction AME treated cells

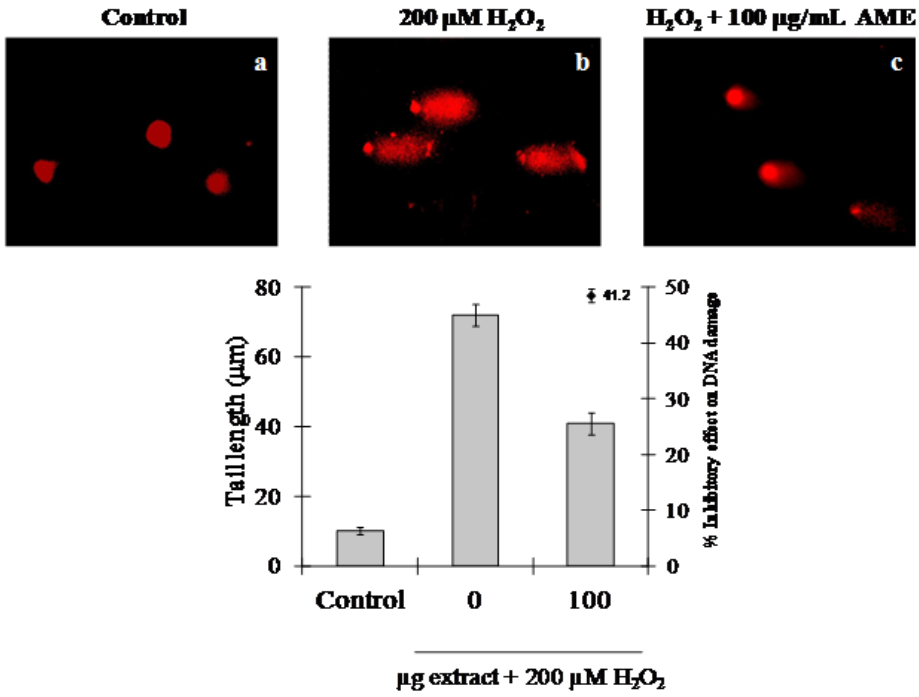

Figure 2B: The protective effect of *A. racemosus* on H_2O_2 induced DNA damage of C2C12 murine muscle cells by single cell gel electrophoresis assay. A. control cells B. 200 µM H_2O_2 treated cells C. 200 µM H_2O_2 + 100 µg/reaction AME treated cells.

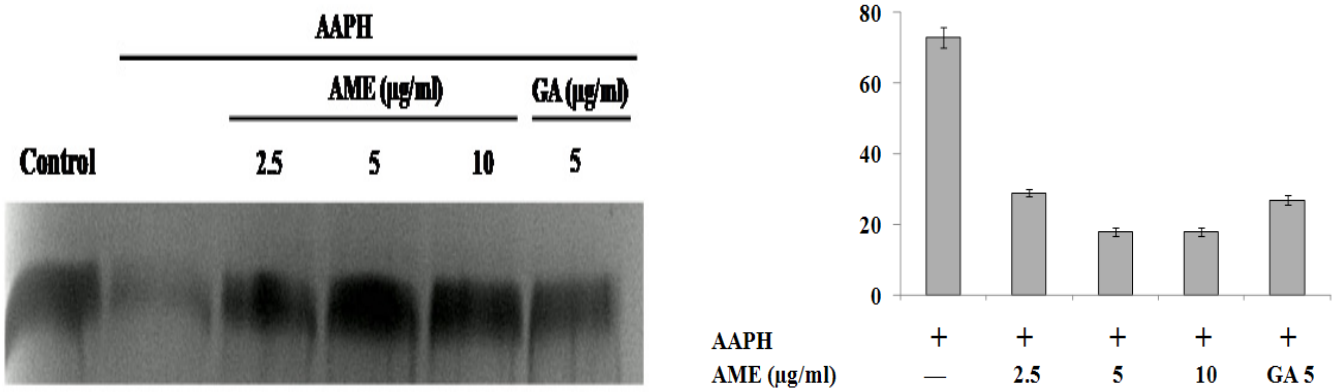

Figure 3: The protective effect of *A. racemosus* on AAPH induced protein oxidation of BSA analysed by polyacrylamide gel electrophoresis. The densitometric analysis of protein oxidation by NIH Image J software.

as shown in (Figure 2a and 2b), which was found to be 85 µm and 72 µm respectively with H_2O_2 challenge. Whereas pretreatment of HT29 and C2C12 cells showed 56.8% and 41.2% DNA damage inhibitory activity respectively. These observations demonstrates the protective effect of *A. racemosus* against colon and muscle cell damage.

AME protects AAPH induced protein oxidation

The protective effect of methanolic fraction of *A. racemosus* was analyzed by BSA induced protein oxidation assay against an azo compound AAPH which generates peroxyl radicals. After the reaction the densitometric pattern analysis of individual protein samples ran by polyacrylamide gel electrophoresis showed that AAPH induces 75% protein oxidation. Whereas the AAPH induced protein oxidation was dose dependently inhibited by pretreatment of BSA with *A. racemosus* (Figure 3).

Lipid peroxidation inhibitory activity of AME

The lipid peroxidation activity of AWE/AME was found to be IC_{50} of 514 ± 23.75/437 ± 22.7 µg/mL respectively, which further confirms that methanolic fraction is a potent lipid peroxidation scavenger compared to that of water fraction of *A. racemosus* (Table 1).

DISCUSSION

Herbs have been used in traditional food system due their nutritional and health benefits. *Asparagus racemosus* is a well known food plant consumed in most parts of the world. *A. racemosus* roots, the edible part of the plant are used in salads, vegetable dishes and soups. It has gained a lot of significance in Indian traditional system of medicine. *A. racemosus* possess an array of compounds that include polyphenols, flavonoids, oligosaccharides, aminoacids, sulphur containing acids and steroidal saponins.[18] these compounds have been reported as radical scavengers due to their antioxidant activity.[19]

Phenolic and flavonoid compounds are reported as antioxidant principles which can alleviate various stress induced disorders such as diabetes, cardiovascular diseases and cancer.[20] It possesses polyphenols such as quercetin-3-glucoronoids, racemosol,[21,22] and flavonoids such as kaempferol, rutin, hyperoside and isoflavone; 8-methoxy-5, 6, 4-trihydroxy isoflavone-7-0-beta-D-glucopyranoside.[2,23,24] Earlier reports demonstrated that these compounds possess anti-inflammatory, antiosteoclastogenic, cytotoxic, immunomodulatory, anti-cancer and antibacterial activities.[25-27]

Herbal extracts have been used as food additives to add health benefits along with nutritional supplementation, these are called functional foods. Hence there is a growing interest across the globe in the commercialization of health foods. The health benefits of food additives and their phytochemicals has to be investigated to give a clear idea about the functional properties of foods.[28] described the add-on benefits of functional foods against wide range of radical mediated cell damage. The radical scavenging ability of *A. racemosus* was evaluated by an array of free radical assays such as DPPH, metal chelating, ABTS, NO, hydroxyl radical scavenging activities. These assays are based on the principle of free radical generation.[1,29] Here we observed that the methanolic fraction of *A. racemosus* exerts potent radical scavenging over water fraction which is due to its hydrogen ion donation, electron transfer, chain termination and nitrite ions reduction activities. This confer that the recovery and composition of phytochemicals varies based on the polarity of the solvents used for the extraction process. The functional properties of extracted solvents also differ based on the composition of phytochemicals recovered.

The macromolecules such as DNA, proteins and lipids damage have been observed in diabetics, cancer and in ageing.[30-32] Several chemicals/radicals were used to evaluate their toxicity at cellular level and the protective activity of functional foods against the toxicity was evaluated routinely by comet assay.[33,34] In our present study the protective effect of *A. racemosus* against H_2O_2 challenged DNA in HT29 human colon, C2C12 mice muscle cells and pRSET A plasmid was analyzed by single cell gel electrophoresis assay and agarose gel electrophoresis. The results demonstrate that AME exerts DNA damage protective effects. Similarly *A. racemosus* also showed protective effects against AAPH induced oxidation of bovine serum albumin protein. The observed results are in accordance with our recent study which shows that Cyperus rotundus exhibits DNA damage and protein oxidation inhibitory activity against H_2O_2/AAPH induced oxidation of macromolecules.[35]

Malonaldehyde (MDA) is a degradation product that is generated by oxidative degradation of polyunsaturated fatty acids in the cell membranes which is an important cause for cellular damage and cell membrane destruction.[36] Estimation of lipid peroxidation has been used as a marker for the oxidative stress. The reaction of MDA with TBA has been widely adopted as a sensitive assay for the lipid peroxidation.[37] In our present study we found that AME significantly inhibits the peroxidation of lipids. Our observed antilipidperoxidation activity is in line with earlier report which also evaluated the lipid peroxidation inhibitory activity of Withania somnifera root extracts.[38]

CONCLUSION

The present study demonstrates that methanolic extract is a potent scavenger of free radicals over water extract of *A. racemosus* as analyzed by an array of *in vitro* antioxidant assays. The methanolic fraction showed inhibitory activity against H_2O_2 induced plasmid DNA damage and AAPH induced protein and lipid peroxidation. Further *A. racemosus* methanolic extract also inhibited H_2O_2 induced human colon and murine muscle cell DNA damage as verified by single cell gel electrophoresis assay. Overall our results demonstrate the antioxidant and macromolecule damage protective effects of *A. racemosus* methanolic extract. However, further phytochemical analysis and *in vivo* studies are required to identify the specific compounds and to better clarify the biological activity of *Asparagus racemosus*.

CONFLICT OF INTEREST

We declare that we do not have any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. HV Batra, Director, Defence Food Research Laboratory, Mysore, for providing all the necessary facilities, constant guidance and encouragement during this investigation.

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Free Radical Scavenging and Cytotoxic Potential of *Celosia argentea*

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ABSTRACT

Introduction: Oxidative stress due to reactive oxygen species often leads to pathogenesis of chronic diseases such as cancer. Research states that a diet rich in polyphenols renders many health benefits by scavenging such harmful reactive species. *Celosia argentea* (Amaranthaceae), a common weed in India has been reported as a potential source of cheap, natural antioxidants due to its phenolic abundance. In this research work efforts were made to identify and screen the phenolic rich fractions of *Celosia argentea* for their antioxidant and anticancer potential. **Materials and Methods:** Various solvent fractions with increasing polarity were subjected to total phenolic content, followed by antioxidant assays- DPPH, ABTS and anti proliferative assays- Brine shrimp Bioassay, Antimitotic and MTT assays. **Results:** IC_{50} value of methanolic fraction for DPPH assay was statistically significant (26.25; ***P<0.001) when compared with ascorbic acid (12.50; ***P<0.001). Also TEAC values for methanolic fraction and BHT (standard) for ABTS assay were similar (2.1; ***P<0.001) Methanolicfraction at 400 μg/ml exhibited strong cytotoxicity (9.0 ± 0.81; ***P<0.001) against brine shrimps comparable to Methotrexate at 50 μg/ml(10; ***P<0.001) and significantly reduced mitotic index from 96.8 to 38.0 (***P<0.001) which was further confirmed by MTT assay where IC_{50} value of methanolic fraction for SiHa and MCF-7 cells was found to be 28 μg/ml with no cytotoxicity to normal cells proving its anticancer potential. **Conclusion:** This research proves antioxidant and anticancer potential of phenolic rich fraction of *Celosia argentea* and suggests it to be useful in cancer management as antifroliferative, chemo preventive and in cancer chemotherapy induced immune suppression and oxidative stress.

Key words: Antioxidant, Brine shrimp, Mitotic index, MTT, Phenolics.

INTRODUCTION

Imbalanced metabolism and excess reactive oxygen species (ROS) generation end into development of oxidative stress leading to range of disorders such as cancer, diabetes, atherosclerosis, cardiovascular diseases, Alzheimer's, Parkinson's disease, aging and many other neural disorders. Toxicity of free radicals contributes to proteins and DNA injury, inflammation, tissue damage and subsequent cellular apoptosis. Antioxidants are now being looked upon as persuasive therapeutic as they have capability to combat by neutralizing free radicals.[1]

As the natural antioxidant defense mechanism becomes inefficient, dietary intake of antioxidant is important to replenish and regenerate antioxidants that are responsible for removal of free radicals, scavenging ROS or their precursors, and binding metal ions needed for catalysis of ROS generation.[2] Synthetic antioxidants like BHT and BHA have their accompanied unavoidable side effects like radio-sensitization, toxicity of other chemicals, mutagenicity, and tumor formation from chemical carcinogens.[3] Hence, there is a need to explore the nature and screen the medicinal plants as potent antioxidants. It is found that dietary polyphenols obtained from various herbs, spices, fruits and vegetables are found to possess anticancer effects via

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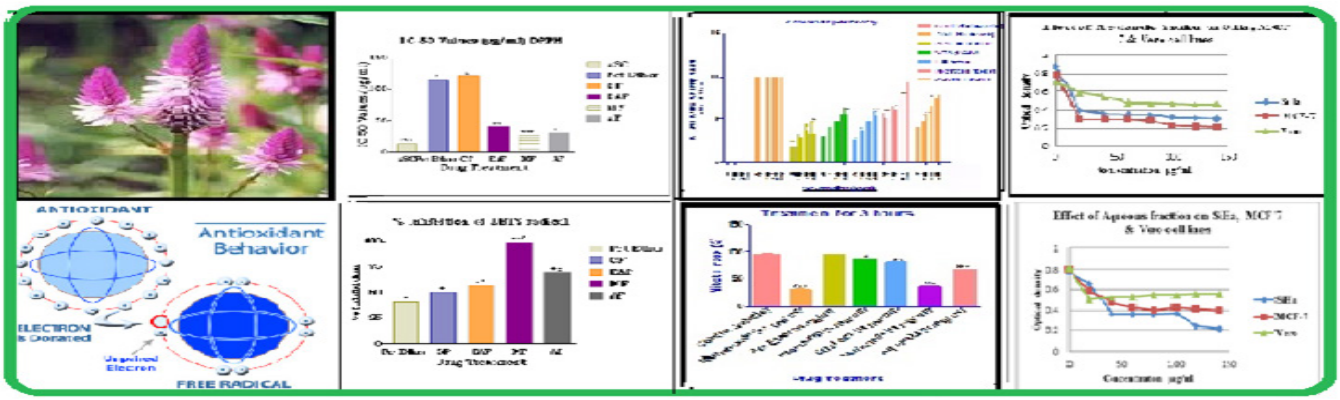
DOI: *10.5530/pj.2015.3.8*



Graphical Abstract

a variety of mechanisms such as removal of carcinogenic agents, modulation of cancer cell signaling and antioxidant enzymatic activities, and induction of apoptosis and cell cycle arrest.[4]

Celosia argentea Linn. Belonging to family Amaranthaceae is a common weed in India found in the bajara fields widely cultivated as an ornamental plant, especially in Southern Europe

The entire plant is used in treatment of ulcers, piles, bleeding nose, inflammation, gynecologic disorders, mouth sores, eye diseases, glandular swellings, eczema, constipation and as an aphrodisiac.[5,6] The seeds are used in the treatment of blood diseases, diarrhea and the roots are well known for their anti-diabetic activity.[7] The *in vitro* and *in vivo* antioxidant activity of the plant is reported to be due to abundance of phenolics making *Celosiaargentea* as a potential source of cheap, natural antioxidants.[8]

Thus, the main objective of research work was to identify and separate the polyphenolic rich fraction using various solvents and subject these fractions to screen its antioxidant and anticancer potential using different assays.

MATERIAL AND METHODS

Plant Material

Fresh whole plant material of *Celosia argentea* Linn was collected from Bhor region, district- Pune, Maharashtra and was authenticated at the Botanical Survey of India (BSI), Pune; (Registration number-BSI/ WC/ Tech./2007) (BSI/ WRC/ Tech./2011).

Extraction

The separated, cleaned and powdered aerial parts were subjected to defatting using n-hexane followed by exhaustive successive solvent extraction using solvents with ascending order of polarity. The extracts obtained were concentrated by using rotary vacuum evaporator and preserved in vacuum desiccator for further pharmacological screening.[9]

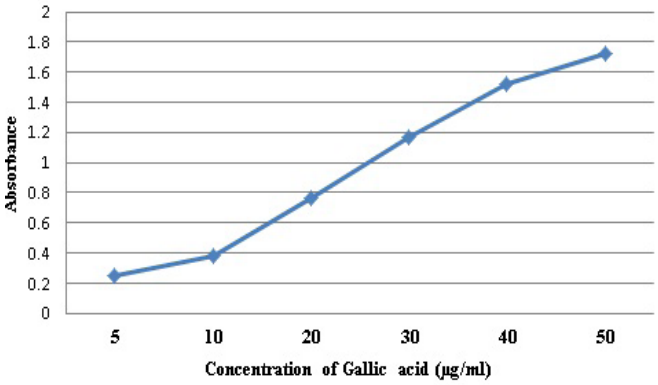


Figure 1: Concentration response curve for gallic acid at 765 nm

Estimation of Total phenolic content

Samples were measured for total phenolic content colorimetrically using the Follin C method with modification. A 100 µl of extract was mixed with 0.5 ml Follin C reagent (diluted 10 times with distilled water). The solution was added with 7 ml of distilled water and allowed to stand at room temperature for 5 minutes. Then, 1.5 ml sodium bicarbonate (60 mg/ml) solution was added to the mixture and left at room temperature in dark place for 2 hours. Absorbance was read at 765 nm against blank using UV-Visible spectrophotometer (Perkin Elmer Lambda 35, USA). A calibration curve was prepared, using a standard solution of gallic acid (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) (Figure 1). Results were expressed as gallic acid equivalents mg (GAE)/100 g.[10]

Pharmacological Investigations of Extracts

In vitro Antioxidant Activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) Assay[11]

A solution of 3.3 mg DPPH in 100 ml methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02 - 0.1 mg of the extract. The reaction mixture was vortexes thoroughly and left in the dark at 25°C for 30 min. The antioxidant oninteraction with DPPH, both transfer electron or hydrogen atom to DPPH, thus neutralizing thefree radical character and convert it to 1, 1-diphenyl-2-picryl-hydrazine. The absorbance ofthe mixture was measured at 517 nm. BHT was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\% \text{ Radical Scavanging Activity} = Abs_{control} - Abs_{sample} / \times 100$$

$Abs_{control}$

Where $Abs_{control}$ is the absorbance of DPPH radical + methanol;

Abs_{sample} is the absorbance of DPPH radical + sample extract/standard.

ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) scavenging activity

The stock solutions included 7 mM ABTS solution and 2.4 mM potassium per sulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantitiesand allowing them to react for 12 hours at room temperature in the dark. The solution was thendiluted by mixing 1 ml ABTS + solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS+ scavenging capacity of the extract was compared with that of BHT and percentage inhibition was calculated using the same equation as that for DPPH method.[12]

In vitro anticancer activity

Cytotoxic Evaluation by Brine shrimp bioassay[9]

In this test, Brine Shrimp *(Artemiasalina)* eggs were hatched in artificial sea water (3.8 g sea salt /liter of water). Generally the samples of test drugs for the experiment are prepared in methanol solution, which acts as a control vehicle. The sample tubes were previously prepared by dissolving specific concentration of test drug to prepare different dilutions followed by evaporation of methanol. After 48 hours of incubation, 10 Brine Shrimps were transferred to each sample tube using Pasteur pipette and artificial sea water was added to makeup the final volume up to 5 ml. Survivors were counted after 12 and 24 hours. Control vials were prepared using methanol only. Three replicates were prepared for each concentration of test drug.

Antimitotic activity[13]

The antimitotic activity was evaluated using the meristematic cells of onion *(Allium cepa)* root tips. The *Allium cepa* roots were sprouted in tap water at room tempatature. When the roots were about 5 mm long,the onion bulbs (five each) were paced on beakers containing the pet ether, chlorofor, ethyl acetae, methanolic and aqueous fractions (10 mg/ml) such that the roots were immersed in the extract. Distilled water and Methotrexate (0.1 mg/ml) were used as control and standard respectively. One hour latter the root tips were cut and transferred to fixing solution acetic acid(45%): ethanol (95%) in the ratio of 1:3v/v (10-12 hrs) followed by warming the root tips in 1 N hydrochoric acid in oven at 50^0C for 15 min. The root tips thus treated were then stained with carmine red stain were observed after a duration of one and three hours.

The slide was then scanned under the microscope to record the number of cells in each stage of cell-division and the mitotic index was caculated using the following formula as per the standard procedure. MI was determined by examination of 500 cells per slide, using 3-5 root tips from each bulb. The slides were examined from right to left, up end down, and the first 500 cells were scored for MI frequency

Standard formula

$$\text{Mitotic index} = \frac{\text{No of dividing cells}}{\text{Total no of cells}} \times 100$$

MTT Assay

The MTT-assay is based on the conversion of the tetrazolium salt MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) to blue formazan which depicts cell viability. Color development in terms of optical density was measured spectrophotometrically at 540 nm and documented by ELISA reader at IRSHA (Bharati

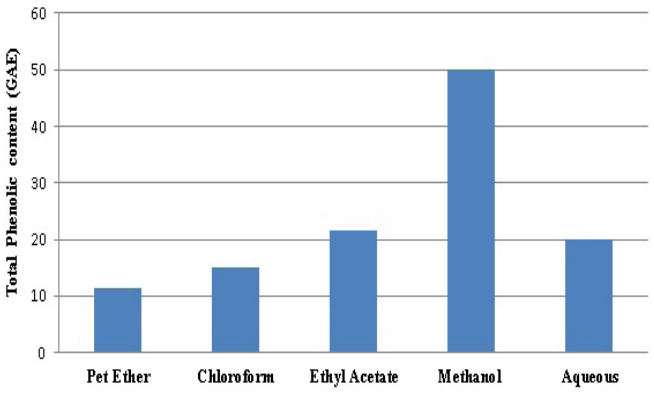


Figure 2: Total phenolic content of various fractions (GAE)

Vidyapeeth University, Pune).[14] Optical density values were converted to % cell viability to get the respective IC_{50} values for SiHa, HCT-15, MCF-7 and Normal monkey kidney Vero cells.[15]

Statistical Analysis

All the results were analyzed using one way ANOVA followed by Dennett's t test.

RESULTS AND DISCUSSION

Total Phenolic Content

The total phenolic content of various fractions of *Celosia argentea*was found in the range of 12-50 µg/ml (GAE/g) with methanolic fraction showing the highest (50 µg/ml;GAE/g) phenolic content (Figure 2).

DPPH Assay

The methanolic fraction of C*elosia argentea* was able to significantly reduce the stable DPPH radical to the yellow-colored diphenyl picryl hydrazine complex indicating potential free radical scavenging activity. The scavenging effect of various fractions and ascorbic acid, measured in terms of IC_{50} value (µg/ml) was observed in the following order: Ascorbic acid (12.50) (***P<0.001) > Methanolic fraction (26.25) (***P<0.001) > Aqueous fraction (30.00) (**P<0.01) > Ethyl acetate fraction (40.00) (**P<0.01) > Chloroform fraction (120.52) (*P<0.1) > Pet Ether fraction (115.00) (*P<0.1) fraction (Figure 3).

ABTS Assay

The TEAC value reflects the relative ability of hydrogen or electron-donating antioxidants to scavenge the ABTS radical cation compared with that of Trolox. The standard curve was linear between 0.5 and 2.0 mM of Trolox (Figure 4) As depicted in (Figure 5), Methanolicfraction showed maximum percentage inhibition of ABTS radical (99.17 %), with a TEAC value of 2.1 (***P<0.001) comparable with a standard antioxidant; BHT (2.1) (***P<0.001) (Figure 5).

Both the antioxidant assays clearly indicate that the Methanolicfraction of *Celosia argentea* possesses significant and concentration dependent free radical scavenging activity equipotent to the standard antioxidants such as ascorbic acid and BHT.

Brine Shrimp Bioassay

The number of brine shrimps dead after 12 hours with chloroform, methanolic and aqueous fractions of Celosia (400 mg/ml) was found to be 5.2, 7.0 and 5.0 respectively. The number was insignificant for pet. Ether and ethyl acetate fractions. Whereas the number of brine shrimps dead after 24 hrs was found to be 3.0, 5.5, 5.2, 9.0 and 7.0 for the pet. Ether, chloroform, ethyl acetate, methanolic and aqueous fractions respectively.

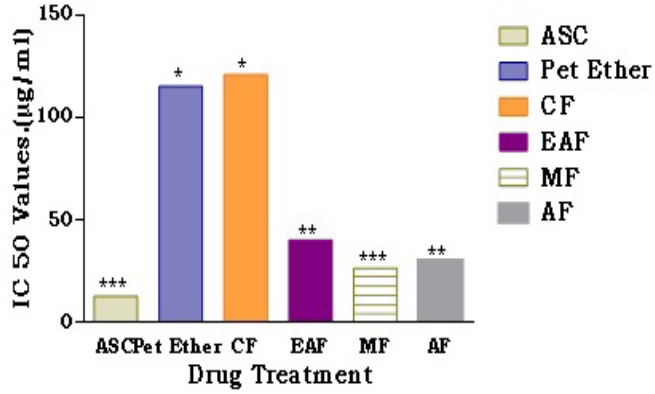


Figure 3: IC_{50} values of various extracts for DPPH Assay

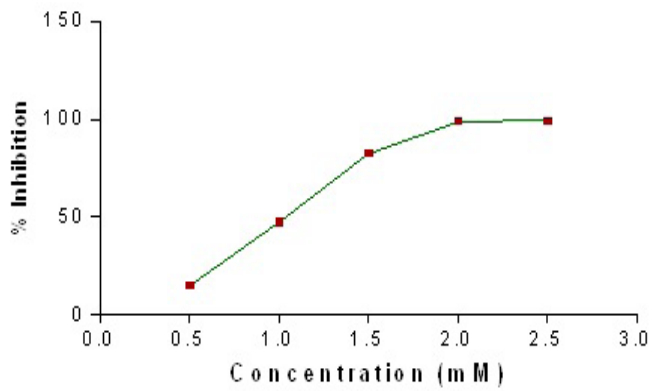


Figure 4: Concentration response curve of Trolox for ABTS at the absorbance at 734 nm

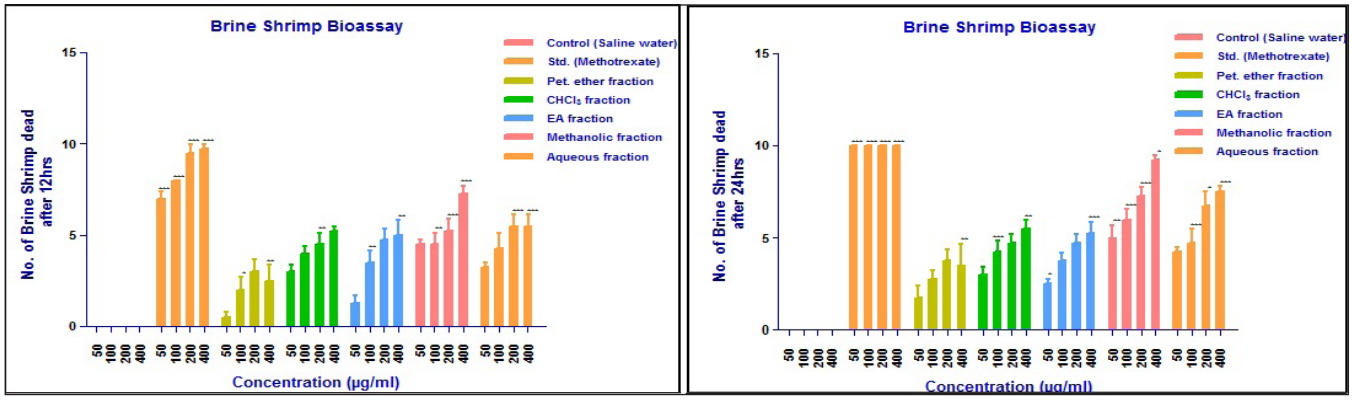


Figure 5: % Inhibition of ABTS radical by various fractions

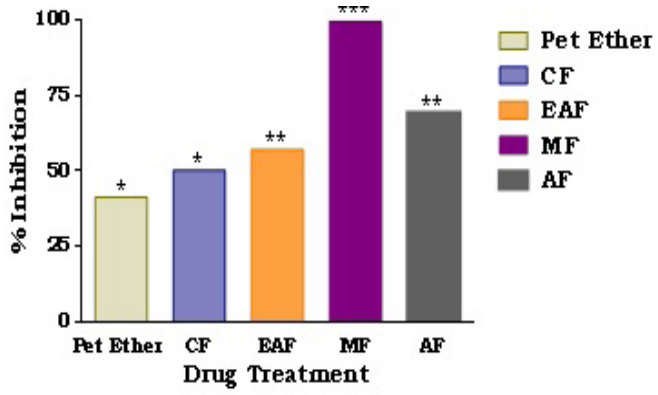


Figure 6: Number of Brine Shrimps dead after 12 and 24 hours.

Further, all the brine shrimps survived in saline water (control) and all the Methotrexate (50 mg/ml) treated Brine Shrimps were found dead (positive control) before 24 hours indicating its potent cytotoxic effect (Figure 6). The above findings indicate that the methanolic fraction at 400 mg/ml showed significant cytotoxic activity (9.0 ± 0.81; ***P<0.001) comparable to Methotrexate (10; ***P<0.001); a standard anticancer drug.

Antimitotic activity

The mitosis in the control group continued to be normal even after 3 hrs. (Figure 7) shows that mitotic indices of all extracts at two different durations were lower than the mitotic index of the control. The methanolic fraction of *Celosia argentea* (10 mg/ml) exhibited significant antimitotic activity in terms of reduction in mitotic index from 97.2 to 43.6 (1 hr treatment) and from 96.8 to 38.0 (3 hr treatment). The results obtained were comparable to that of Methotrexate with Mitotic indices of 46.2 (1 hr treatment) and 32.8 (3 hr treatment) (***P<0.001). Pet. ether, chloroform, ethyl acetate and aqueous fractions were found to be insignificant.

MTT Assay

The potential cytotoxic effect of various fractions of *Celosia argentea* was investigated on the viability of SiHa and MCF-7 cell lines by MTT assay. The results summarized in (Figure 8) indicate that methanolic fraction showed potent anticancer activity against both the cell lines with inhibitory concentration of 28 µg/ml (***P<0.001) with no toxicity towards the normal cells than the investigated

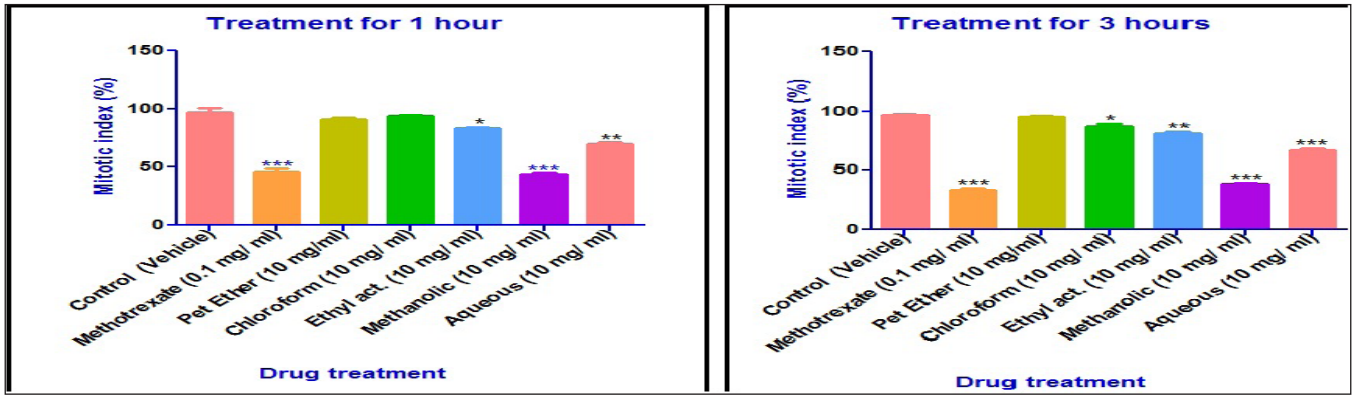


Figure 7: % reduction in Mitotic Index after treatment for 1 and 3 hours

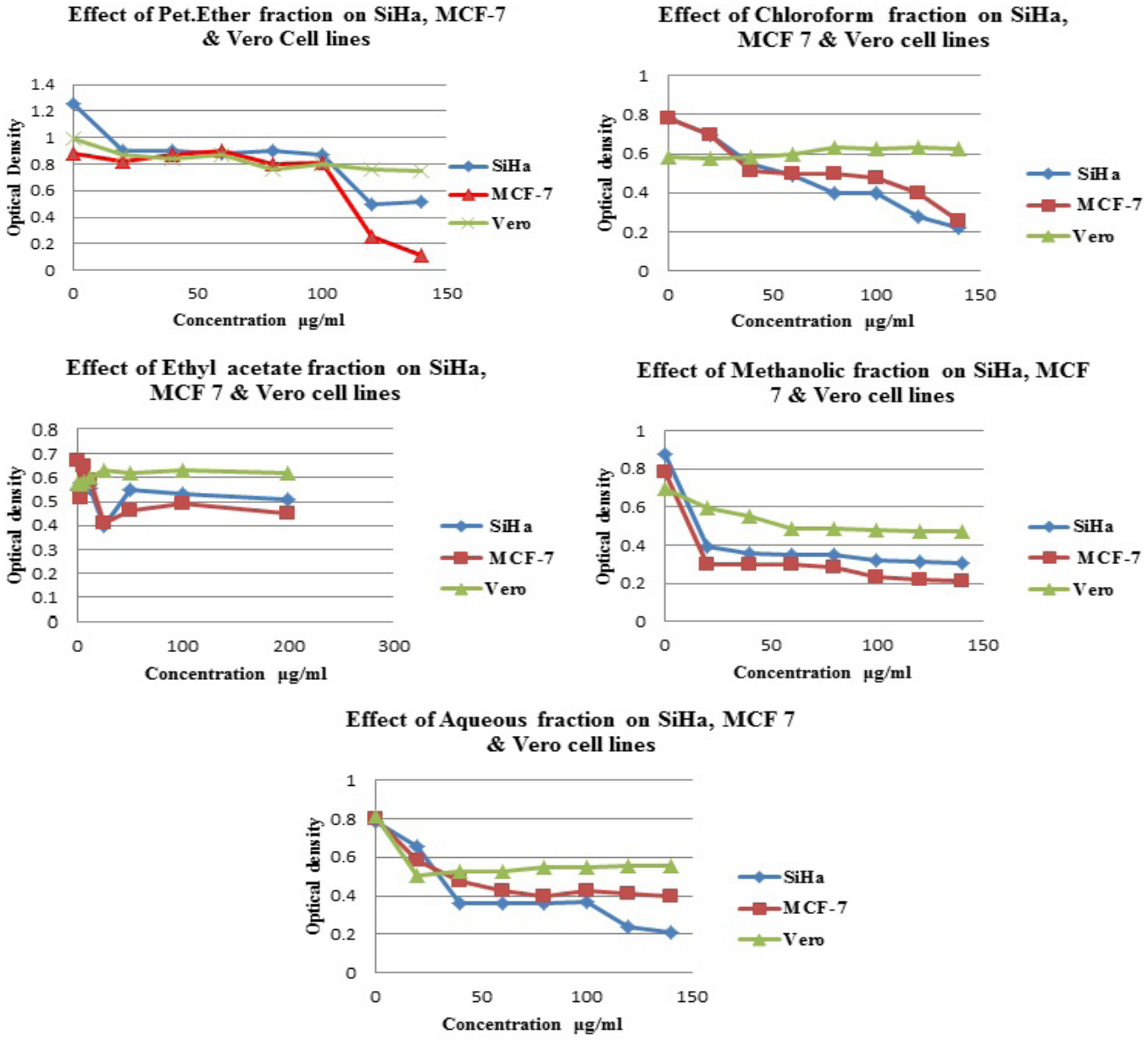


Figure 8: MTT Assay of various fractions

cancer cell lines.

CONCLUSION

The antioxidant potential of methanolic fraction makes it useful in reducing the oxidative stress generated due to reactive oxygen species for combating cancer and cancer chemotherapy induced immunosuppression. Thus, the phenolic rich methanolic fraction of *Celosia argentea* can is used either individually or as a natural adjuvant in cancer therapy along with other chemotherapeutic agents.

ABBREVIATIONS

ROS :Reactive oxygen species
BHA :Butylated hydroxyl anisole
BHT :Butylated hydroxyl toluene
DPPH :2,2-Diphenyl-1-picrylhydrazyl
GAE :Gallic acid equivalent
TEAC :Trolox equivalent antioxidant capacity
ABTS :2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
BST :Brine Shrimp test
MTT :(3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide)
ELISA :Enzyme-linked immunosorbent assay

CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENTS

We extend our deep gratitude to the Principal and Management of Padmashree Dr. D. Y. Patil Institute of Pharmaceutical Sciences & Research for letting us avail their infrastructure facilities and extending their valuable guidance and support.I also thank Management and Principal of Allana College of Pharmacy for their constant encouragement and support.

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Dynamic Comparison of Free Radical Scavenging Abilities of *Hypericum Perforatum L.*, *Herba Verbenae Officinalis*, and *Valeriana Officinalis L.* Extracts

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ABSTRACT

Objectives: The purpose of this research was to compare the dynamic antioxidant activities and the free radical scavenging abilities of three botanical supplements, *Hypericum perforatum L*. (HPL), *Herba Verbena Officinalis* (HVO), and *Valeriana officinalis L*. (VO), which have been reported to effectively treat menopause symptoms. **Methods:** The antioxidant activities of the three supplements were determined by the ferric reducing ability of plasma (FRAP) assay. In addition, their free radical scavenging abilities were studied by ftheir interactions with the stable radicals of 2, 2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). **Results:** The results indicated that the HPL ethanolic extract exhibited greater antioxidant activity (1.2688 ± 0.01 mmol Trolox/g) than the HVO ethanolic extract (1.1686 ± 0.043 mmol Trolox/g) and the VO ethanolic extract (0.2579 ± 0.0031 mmol Trolox/g) as compared to Nilestriol (0.0026 ± 0.0012), a positive control agent in the experiment. Moreover, the HPL extract showed remarkable free radical scavenging activity against ABTS•+, and the HVO extract was the most potent against DPPH•. **Conclusion:** These activities may be attributed to the total flavonoid or phenolic acid contents of among these extracts.

Key words: ABTS•+, DPPH•, *Hypericum perforatum L., Herba Verbena Officinalis,* Menopause Oxidative Stress, *Valeriana officinalis L.*

INTRODUCTION

Menopause generally occurs in women between 45 and 55 years of age. This permanent cessation of menses causes ovarian dysfunction and accelerates the decline of endogenous hormones, leading to many psychopathological reactions, such as hot flashes, arteriosclerosis, skin aging, insomnia, depression, etc. Previously, the link between oxidative stress and ovarian hormone secretion has been well documented. Synthetic hormones taken by women to restore natural ovarian hormone levels have been regarded as a "double-edged sword," since they increase the risk of cardiovascular diseases and breast cancer.[1-3] As an alternative therapy, some plant extracts (phytoestrogens) have been confirmed as powerful radical scavengers not only *in vitro* but also in rodents and human trials.[4,5] Recently, the correlation between female metabolism disorders, especially those causing undesirable menopause symptoms, and oxidative stress has been reported; in addition, there has been an increased interest in phytotherapy due to the risk of hormone therapy for menopause symptoms.[4] *Hypericum perforatum L.* (HPL).[6-9][Abdali, 2010 #104; van Die, 2009 #108; Al-Akoum, 2009 #106; Sloley, 2000 #82] *Herba Verbenae Officinalis* (HVO),[10] and *Valeriana officinalis L.* (VO)[11-13] are three traditional herbal supplements that have been used for many years in female metabolic disorder treatments and have been shown to improve

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DOI: *10.5530/pj.2015.3.9*

women's health in experimental and clinical studies. Redox reactions *in vitro* and *in vivo* primarily involve the transfer of electrons and hydrogen atoms.[14] The synthetic stable molecules 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)[15] have been used to investigate the kinetics of radical scavenging reactions.[15-18] The reaction between antioxidants and DPPH free radicals (DPPH•) has a kinetic feature and is widely used to assess the ability of antioxidants to transfer labile protons to radicals.[15] The blue-green free radical cation of ABTS ($ABTS^{\bullet+}$), which is used to assess the ability of antioxidants to receive electrons from radicals, has also been extensively used to characterize antioxidants in solution.[17,18] The purpose of this study was to investigate the dynamic antioxidant activities and the free radical scavenging abilities of HPL, HVO, and VO extracts compared with the positive control Nilestriol, (3-(Cyclopentyloxy)-17alpha-ethinyl-1, 3, 5(10)-estratrien-16alpha, 17beta-diol), an orally available estrogen-type drug that has been often used in hormone replacement therapy to treat long-term side effects due to menopause symptoms.[19] Based on our previous methods,[20] the antioxidant activities of the three extracts and Nilestriol were determined by the ferric reducing ability of plasma (FRAP) assay. The free radical scavenging process was monitored by measuring absorbance as a function of time of reactions of each extract with the radicals $ABTS^{\bullet+}$ and DPPH•. The results of this study will determine whether these plant ethanolic extracts have the capacity to combat oxidative stress to treat undesirable menopause symptoms.

MATERIALS AND METHODS

Chemicals and Materials

Nilestriol (97% pure) and the stable free-radical scavengers ABTS (95% pure) and DPPH (95% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The total antioxidant capacity assay kit was purchased from Beyotime (Jiangsu, China). The standards rutin and gallic acid, and other chemicals and reagents (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Extracts preparation

Entire herbs of HPL, aerial parts of HVO, and roots/rhizomes of VO were collected from Shanxi, Hubei, and Gansu provinces in China, respectively. The crude plants were washed thoroughly with distilled water, dried under shade, and ground into powders that could pass through a sieve of pore size 0.5 mm. Each dry powder (100 g) was extracted with 1000 mL of 95% ethanol, and this process was repeated three times at room temperature(Each 1 g of extract was equivalent to 100 mg of dry herb).Then, the ethanolic extracts were filtered, and the filtrates were combined. Finally, ethanol was removed by vacuum distillation using a rotary evaporator at a reduced pressure and no more than 50°C, and the extracts were subsequently lyophilized and stored at -20°C until use.[21]

Botanical composition analysis

According to preclinical and clinical trials, flavonoids and phenolic acid are the major phytochemical compound types used for menopausal symptom relief treatment. Therefore, the total flavonoid and phenolic contents were determined via a UV-2501PC UV-Vis Recording Spectrophotometer (SHIMADZU, Japan). The total flavonoid content was determined by an aluminium chloride colorimetric method.[22] The absorbance was determined at 403 nm. The total flavonoid content was expressed as mg of rutin equivalents (RE)/g of dry plant powder (DP) that was calculated by the calibration curve equation: $y=24.5x + 0.0137$ ($r=0.9998$), where y and x represent absorbance and RE, respectively (the standard rutin concentration ranged from 2 to 24 μg/mL). The total phenolic acid content was determined using the Folin-Ciocalteu method.[23] The absorbance was measured at 500 nm. The standard curve of the method was $y=2.5757x - 0.006$ ($r=0.9990$), where y is absorbance and x is gallic acid equivalents (GAE; the standard gallic acid concentration ranged from 25 to 250 μg/mL). The total phenolic acid content was expressed as mg of GAE/g of DP based on the above calibration curve equation.

Antioxidant activity assay

To measure the total antioxidant activity of Nilestriol (positive control), HPL, HVO, and VO extracts, the FRAP assay, developed by Benzie *et al.* was used. Standard solutions of $FeSO_4 \bullet 7H_2O$ (0.625, 1.25, 2.5, 5, and 10 mM) were prepared in deionized water. Each extract was diluted 2:98 (v/v) in 2,3,5-triphenyl-tetrazolium chloride (TPTZ), and the experimental procedure followed that of Benzie *et al.*[24] The FRAP was defined as the concentration of $FeSO_4$ that provided an antioxidant activity equivalent to 1 mM of the substance under investigation, and the antioxidant activity was given in units of mmol Trolox/g of extract.

Dynamic process of scavenging ABTS•+

A stock solution of $ABTS^{\bullet+}$ was prepared according to the

procedure of Re *et al.*,[25] with some minor modifications. The ABTS$^{\bullet+}$ solution was produced by incubating 2.8 mM potassium persulfate with 7 mM ABTS in water for 12 to 16 h. The solution was kept in the dark until use, and it was used within 24 h. The stock solution was diluted 125-fold with phosphate-buffered saline (PBS, 5 mM sodium phosphate, pH 7.2) to obtain a standard solution that contained approximately 36 μM ABTS$^{\bullet+}$ and 18 μM ABTS. The HPL, HVO, and VO extracts and control reagents were added as described, and the reaction was followed by monitoring the change in the absorbances of ABTS and ABTS$^{\bullet+}$ by a Cary 50 spectrophotometer (Varian) using Cary WinUV software.

Determining the dynamic change of ABTS•+ and ABTS

The absorbance of the reactions between ABTS$^{\bullet+}$ and HPL, HVO, or VO extract was measured and compared with the PBS control (5 mM, pH 7.2). After reading the initial absorbance, each extract was diluted 2:98 (v/v) in ABTS$^{\bullet+}$ stock solution. (The final concentrations of HPL, HVO, and VO extracts were 30 μg /mL). The reaction of each extract with the standard ABTS$^{\bullet+}$/ABTS solution was followed by measuring the absorbance at 415 nm and 340 nm using absorbance scanning on a Cary 50 spectrophotometer (Varian) using Cary WinUV software. The concentrations of ABTS and ABTS$^{\bullet+}$ were calculated using the following equations: $\varepsilon_{340} = 4.8 \times 10^4/M/cm$ and $\varepsilon_{415} = 3.6 \times 10^4/M/cm$, respectively.[18] The initial reaction rates of ABTS$^{\bullet+}$ consumption for 0 to 600 s were determined.

DPPH radical scavenging assays

A working solution of DPPH$^{\bullet}$ was prepared using a 0.1 mM DPPH solution in methanol, and the solution was kept in the dark before use. The proton transfer reactions between each extract (HPL, HVO, and VO) and DPPH$^{\bullet}$ were monitored in real time by a Cary 50 (Varian) spectrophotometer using Cary WinUV software. The DPPH$^{\bullet}$ concentration was calculated using the following equation: ε_{515}=11,240/M/cm.[26] The reaction between DPPH$^{\bullet}$ and each extract was observed by measuring the absorbance from 260 to 700 nm of a reaction mixture of extract in DPPH$^{\bullet}$ solution (5:95 (v/v)). Each extract or control reagent (5 mM PBS, pH 7.2) was added as described for each experiment, and the reaction was followed by monitoring the change in the absorbance of DPPH$^{\bullet}$ at 515 nm. Time-resolved absorption spectra were collected with times ranging from 0 to 600 s.

Table 1: Total phenolic acid and flavonoid contents of the dry plant extracts (n = 3).

Plant extract	Total Flavonoid (mg RE/g DP)	Total Phenolic acid (mg GAE/g DP)
HPL	86.8 ± 3.4	47.3 ± 1.4
HVO	14.9 ± 0.3	80.7 ± 2.6
VO	4.2 ± 0.3	22.8 ± 0.8

Total flavonoid content is expressed as mg of rutin equivalents (RE) per g of dry plant powder (DP). Total phenolic acid content is expressed as mg of gallic acid equivalents (GAE) per g of DP. Data are represented as means ± standard error (SE) of three replicates.

RESULTS

Botanical composition analysis

The contents of total flavonoids and total phenolic acid among HPL, HVO, and VO extracts are listed in Table 1 Clearly, HPL had a greater total flavonoid content among these extracts, while VO contained the least. On the other hand, the highest content of total phenolic acid was observed in HVO, followed by HPL and VO.

Antioxidant activity determination

The FRAP values of HPL, HVO, and VO extracts compared to Nilestriol were determined in order to evaluate their antioxidant activities. Their reducing abilities were measured spectrophotometrically by their absorbance at 593 nm, and the data were summarized. According to the standard reaction curve, the mean antioxidant activities (represented by the FRAP values) of Nilestriol, HPL, HVO, and VO extracts were 0.0026±0.0012, 1.27±0.010, 1.17±0.043, and 0.258±0.0031 mmol Trolox/g (±SE), respectively. Thus, HPL, HVO, and VO extracts had a low but significant level of antioxidant activity.

Reaction between ABTS•+ and HPL, HVO, and VO extracts

The kinetics process is important for the free radical scavenging activity determination of Nilestriol, HPL, HVO, and VO extracts; thus, ABTS•+ was used to evaluate the antioxidant capacities.[27] In our experiments, the ABTS•+ concentration was significantly reduced by each extract studied (Figure 1A,1B,1C,1D). The spectra were collected from 290 nm to 900 nm as a function of time, and they revealed a decrease in the ABTS•+-specific absorbance at 415 nm and a corresponding increase in the ABTS-specific absorbance at 340 nm. ABTS•+ alone and Nilestriol exhibited a minor amount of auto-scavenging activity (Figure 1E); however, the HPL, HVO, and VO extracts significantly increased this activity in a time-dependent

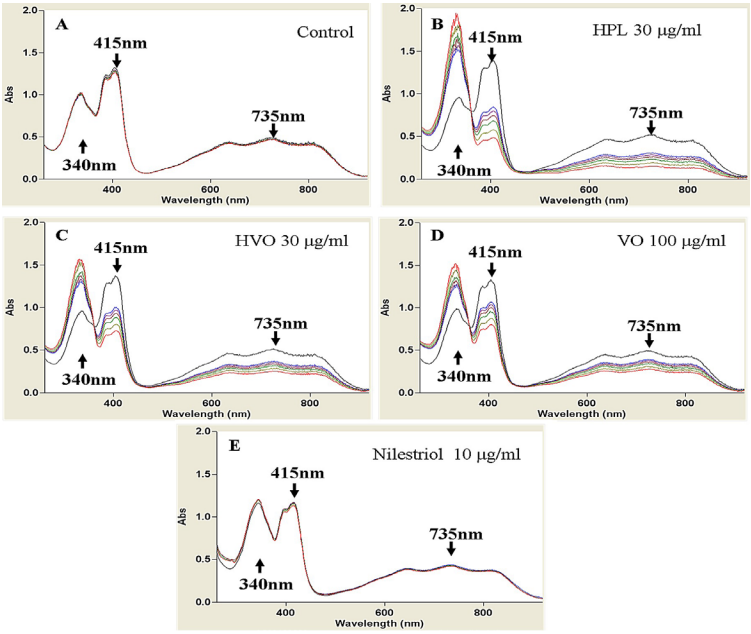

Figure 1: Reactions between ABTS•+ and PBS control (A), HPL (B), HVO (C), VO (D) extracts, and Nilestriol (E).

The control and extracts were added to the ABTS•+ solution. The final concentrations of HPL and HVO were 30 μg/ml, and that of VO was 100 μg/ml. The absorption spectra at 340, 415, and 735 nm were read at regular time intervals from 0 s to 600 s.

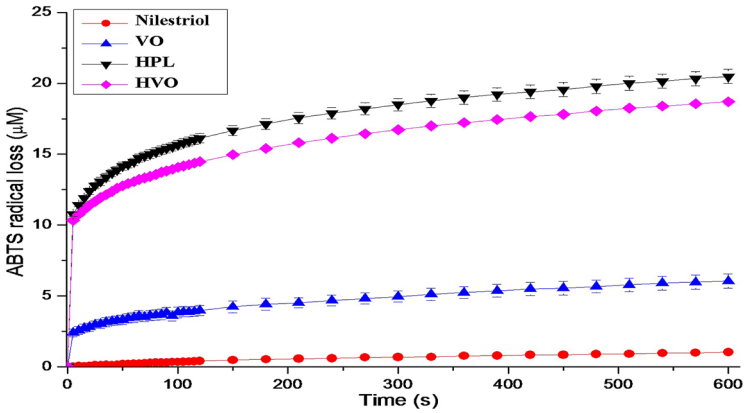

Figure 2: The ABTS•+ dynamic scavenging activities of HPL, HVO, and VO extracts and Nilestriol.

The final concentrations of HPL, HVO, and VO were 30 μg/ml, while that of Nilestriol was 10 μg/ml. Reactions between the standard ABTS•+/ABTS solution and HPL, HVO, or VO extracts or Nilestriol were followed by reading the absorbances at 415 nm and 340 nm. The initial reaction rate and the end of ABTS•+ consumption during the initial rapid reaction phase were estimated as shown in the figure.

manner. Thus, HPL, HVO, and VO extracts have a slow but stable level of dynamic ABTS•+ scavenging activity.

Dynamic process of ABTS formation and ABTS•+ loss

To determine the process of ABTS formation and ABTS•+ loss, the total concentrations of ABTS•+ and ABTS were calculated. Upon addition of each extract to the ABTS•+ solution, the dark green free radicals were converted into the colorless compound ABTS. The amounts of increased ABTS and decreased ABTS•+ were quantified by measuring the increase in the absorbance at 340 nm (ε340 =4.8 × 104/M/cm) and the decrease in absorbance at 415 nm (ε415=3.6 × 104/M/cm). As shown in (Figure 2), ABTS formation and ABTS•+ consumption by Nilestriol, HPL, HVO, and VO extracts occurred in a time-dependent manner.

Reaction between DPPH• and HPL, HVO, and VO extracts

The typical sets of full-band scanning that were recorded after mixing 0.1 mM DPPH• with Nilestriol (0.12 mg/mL), HPL, HVO, and VO extract solutions (0.25 mg/mL, respectively). The reactions were completed within 600 s (Figure 3A, 3B, 3C, 3D, 3E). DPPH• was significantly reduced by HPL, HVO, and VO extracts. The spectra were collected from 260 nm to 800 nm as a function of time, and they revealed a decrease in the DPPH•- specific

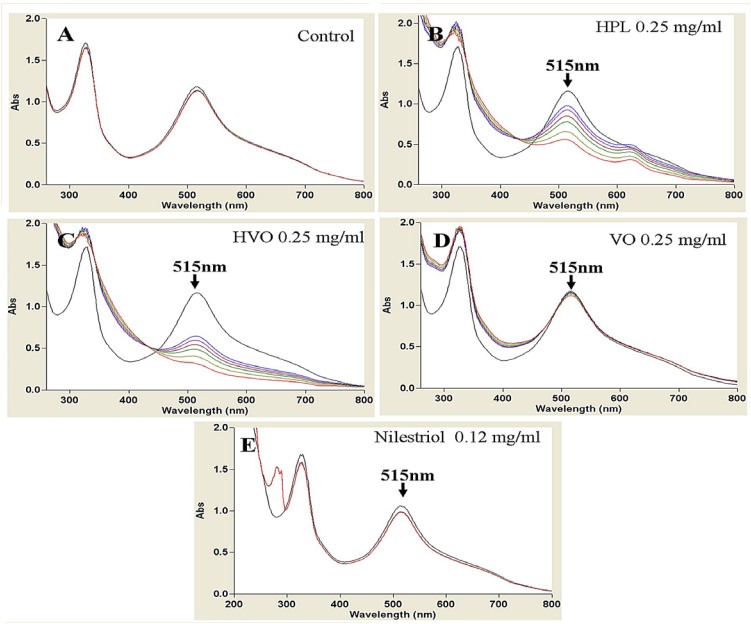

Figure 3: Reactions between DPPH• and PBS control (A), HPL (B), HVO (C), VO (D) extracts and Nilestriol (E).

The extracts or control reagents were added to the DPPH• solution to give an extract final concentration of 0.25 mg/ml and a Nilestriol final concentration of 0.12 mg/ml. The absorption spectra at 515 nm were read at regular time intervals from 0 s to 600 s..

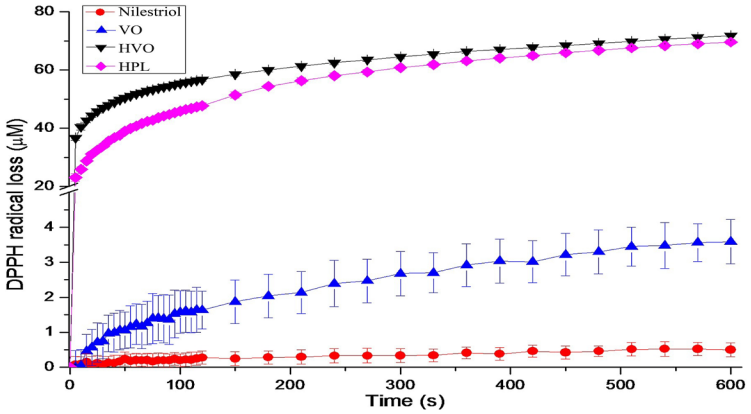

Figure 4: The DPPH• dynamic scavenging activities of HPL, HVO, and VO extracts and Nilestriol.

The final concentrations of HPL, HVO, and VO were 0.25 mg/ml and that of Nilestriol was 0.12 mg/ml. The reactions of each extract with the standard DPPH•/DPPH solution were followed by measuring the absorbance at 515 nm. The initial reaction rate and the end of DPPH• consumption during the initial rapid reaction phase were estimated as shown in the figure.

absorbance at 515 nm. The time profiles of these changes are shown in (Figure 3A, 3B, 3C, 3D, 3E). Thus, HPL, HVO, and VO extracts have a slow but stable dynamic DPPH• scavenging activity.

Dynamic process of DPPH• loss

To identify the process of DPPH• loss, the DPPH• concentration was calculated. Nilestriol, HPL, HVO, and VO extracts were reacted with DPPH•, which converted the red radicals into colorless compounds. The amount of the reduction of DPPH• was quantified by measuring the increase in the absorbance at 515 nm (ε515=11,240/M/cm). As shown in (Figure 4), DPPH• consumption occurred in a time-dependent manner.

DISCUSSION

The botanical supplements HPL, HVO, and VO have been shown to demonstrate antioxidant and free radical scavenging abilities.[28] Previous research has only investigated the dynamic parameters of their free radical scavenging activities with respect to hydroxyl radical scavenging, so new data are needed for other radicals. VO is widely used for the treatment of anxiety and unrest.[29,30] One in vivo study has demonstrated the free radical oxidation of valerian extract, suggesting that it could be modulated

by psychotropic drugs.[31] Arecent study examined the antioxidant activities of terpenoids from valerian oil using DPPH• scavenging activity and FRAP assays. The results indicated that terpenoid structures scavenged DPPH• and increased the ferric reducing capacity in a concentration-dependent manner.[32] These reports suggest that free radical scavenging is an important physiological function of HPL, HVO, and VO extracts; however, the kinetic process of radical scavenging has not yet been documented.The main findings of our study were that HPL, HVO, and VO extracts all show potent antioxidant activity and dynamic scavenging activities of $ABTS^{\cdot}+$ and DPPH·. Our current results suggest the following: (1) rapid and stable radical scavenging capacity is an important characteristic of HPL, HVO, and VO extracts, (2) the scavenging activity of HPL, HVO, and VO extracts is time-dependent, and (3) the $ABTS^{\cdot}+$ and DPPH· scavenging ability of these extracts gradually increased during the time course of the reaction, (4)HPL indicated a remarkably high total flavonoid content when compared to HVO and VO, which showed a negative correlation to ABTS•+ scavenging activities. Meanwhile, the total phenolic acid content of HVO was twice and four times greater than HPL and VO, respectively, which highly correlated with its DPPH• scavenging activities. Therefore, these data support the hypothesis that HPL, HVO, and VO extracts may prevent reactive oxygen species-related injuries by scavenging free radicals in the body.

CONCLUSION

According to the assays of ABTS•+ and DPPH•, we found that HPL, HVO, and VO extracts had a slow but stable level of dynamic ABTS•+ and DPPH• scavenging activity. These activities may be attributed to the total flavonoid or phenolic acid contents of among these extracts.

CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Project No. 21375147), the National Municipal Natural Science Foundation of Beijing (Project No. 7142125), the Integrated Drug R&D Platform (No. 2012ZX09301003-001-010) for "the significant creation of new drugs" under major projects of National Science and Technology, and the project of significant creation of new drugs of National Technology (No. 2013ZX09102-016).

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Morphological and anatomical investigations of *Vicia truncatula Fish. ex Bieb.*

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ABSTRACT

Introduction: Micromorphological research of vegetative bodies of *Vicia truncatula Fish. ex Bieb.* as a source of a sum of diosmetine biozides (flavicine) with high antioxidant hepatoprotective and endothelial-protective activities. We have identified the main diagnostic signs of vegetative bodies which can be used when standard documentation on medicinal vegetable raw materials compile. **Materials and Methods:** The objects of the study were collected in North Caucasus (Russia) in June 2013-2014. Materials morphological study herbarium specimens were stored in the herbarium fund the Department of Botany (acronym PGFA). Materials micromorphological study were temporary slides are the root, leaf, stem and flowers. **Results:** The morphological results were compared with the Flora of the USSR. Anatomical characters of leaves and stems of the species were observed to be similar to the usual features of genus Vicia anatomy. All results are supported by photographs. The stomatal apparatus belongs to anomocytic and paracytic types, simple multicellular hairs. **Conclusion:** we would like to note that these investigations are one of the phases of *Vicia truncatula Fish. ex Bieb*. examination and the medicinal plant is considered to be a perspective source of flavicine. The morphological and anatomical research obtained data can be used in standard documentation for medicinal vegetable raw material compiling.

Key words: Anatomy, Morphological and Anatomical investigations, Morphology, *Vicia truncatula Fish. ex Bieb*.

INTRODUCTION

This work is a fragment of the morphological and anatomical studies of promising resurnyh flora of the North Caucasus.[1] The target of our study is morphological and anatomical research of Wicky succise (*Vicia truncatula Fish. ex Bieb*). This species is widespread all over the Ciscaucasia; it is also considered as an endemic of the West and East Ciscaucasia.[2,3]

For the last years, some studies have showed the perspective of Wicky succise use as a source of a biologically active agent. The sum of diosmetine biozides, containing in the herb, tentatively called *flavicine*, has a strong antioxidant effect. It showed high hepatoprotective activity if there is an acute and chronic four-chloride trophopathic hepatitis. It improves anti-inflammatory, antiproliferative, antithrombotic and hypolipidemic functions of endothelium in different pathologic processes including diabetes. It normalizes NO-synthazes activity and blood hemostasis balance as well.[3,5-7]

Raw materials were collected on the south-west slopes of Mashuk Mountain (in Pyatigorsk) in the period from June 2013 to 2014. *Vicia truncatula Fish. ex Bieb.* life form is a perennial, climbing herb with a large quite curved rhizome. (Figure 1) The system of adventitious root is typical; sprouts are elongated, uprighted or quite ascending, which are branched higher of middle. It has unparipinnate leaf, petiolated leaflet. The epiphyll form is oval or elliptical, the top of leaflet has a sharp tip, the edge is integral, and the base is round. The leaf has very narrow stipules. Inflorescences are botryoid and simple; the cluster consists

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DOI: *10.5530/pj.2015.3.10*

Figure 1: Morphological description of *Vicia truncatula Fish. ex Bieb.*

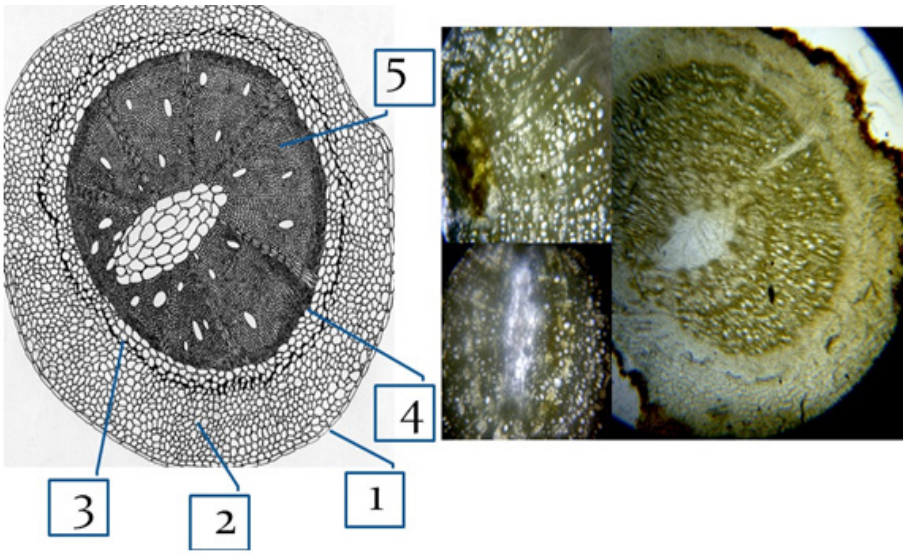

Figure 2: The anatomic structure of *Vicia truncatula Fish. ex Bieb*. root.

of 7-13 flowers. The calyx is campaniform, and the teeth are unequal. The low teeth are lanceolate and theyare shorter than the tube. The high teeth are very short and they are connivent. The color of corolla is primrose and it isn't covered by fibres a lot with size about 13-16 mm. The androceum consists of 10 stamens. Gyneceum monocarpic. The fruits are black elongated beans; they are short-cut to the tops.

MATERIAL AND METHODS

In carrying out morphological studies revealed as morphological characteristics. Micromorphological research of vegetative bodies was carried out with the help of a well-known technique.

RESULTS

The morphological structure of this type is the study of life forms, type of root system, structure of leaves, inflorescences and flowers.

The cross cut of a root

The root has a secondary structure and it consists of two blocks. The first one is an investing tissue and the secondone is a central cylinder. The investing tissue is presented by a periderm. The central cylinder consists of

pericycle, phloem and xylem. Pericycle is presented by parenchymatous cells which are situated on several layers.

The phloem is laid in the form of a ring. It consists of thin-walled cells which are situated very tightly to each other. The cambium settles down inside from phloem in some layers. It presented by little live cells with a rectangular shape. Xylem occupies the primary part of the roots cross cut and consists of thick-walled rounded vessels and parenchymatous cells. (Figure 2)

The cross cut of a stem

The stem has asecondary structure and it consists of three blocks. The first one is an investing tissue, the second one is a cortex and the third one is a central cylinder. The investing tissue ispresented by an epidermis with the collenchyme under the epidermis. The collenchyme is formed by oval cells with irregular thickening of cellular wall. (Figure 3)

The central cylinder consists of sclerenchyme, phloem, cambium, xylem and parenchyme of the pith. The sclerenchyme is placed on the separate area over the phloem. The phloem consists ofsieved tubes and cells companions. The xylem consists of vessels. The parenchyme of the pith consists of big cells with a thin cellular wall.

The cross cut of a leaf plate

The leaf is dorsoventral, amphistomatic. The leaf plate

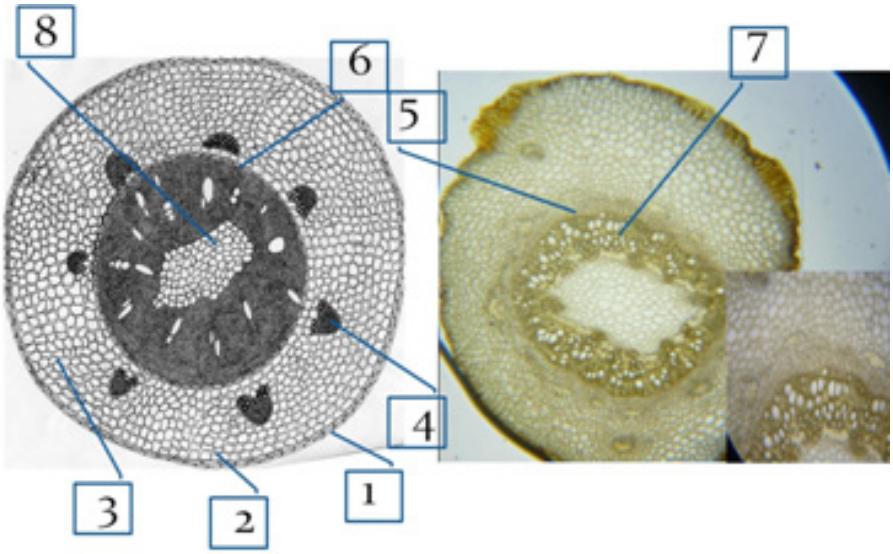

Figure 3: The anatomic structure of *Vicia truncatula Fish. ex Bieb*. stem

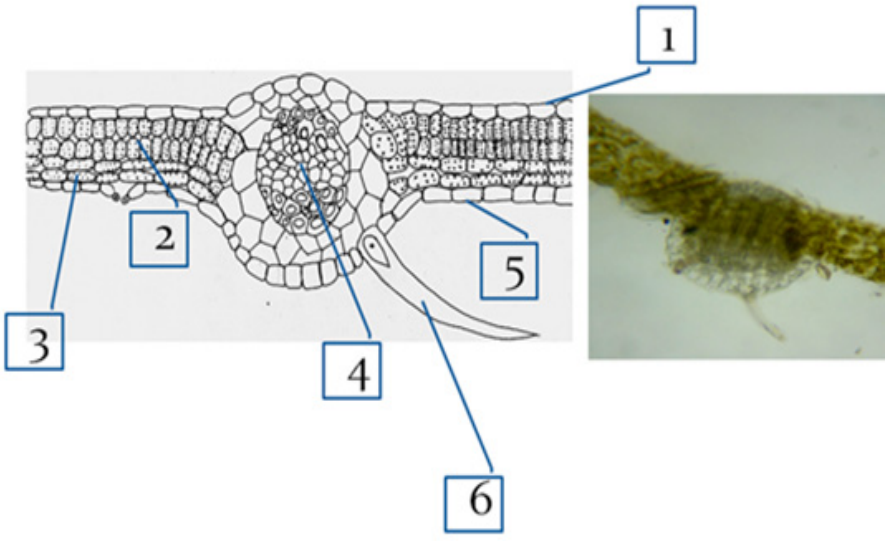

Figure 4: The anatomic structure of *Vicia truncatula Fish. ex Bieb*. leaf

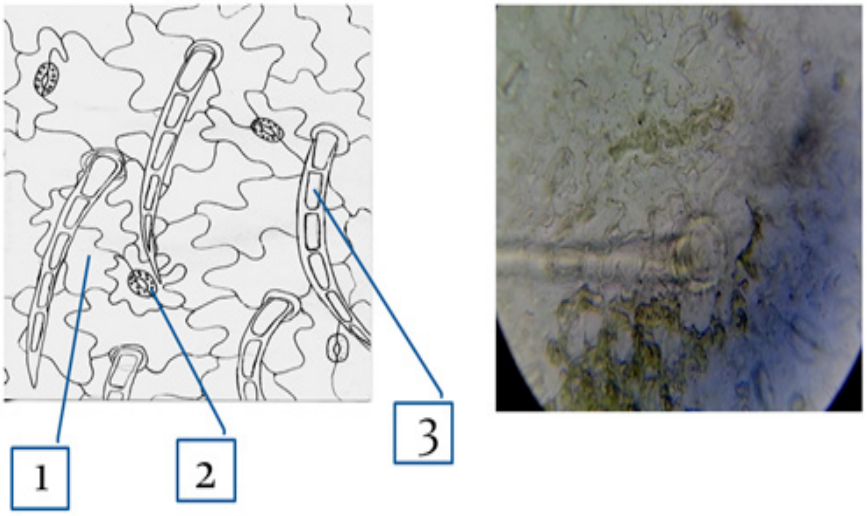

Figure 5: The structure of the lower epidermis of *Vicia truncatula Fish. ex Bieb*. leaf plate

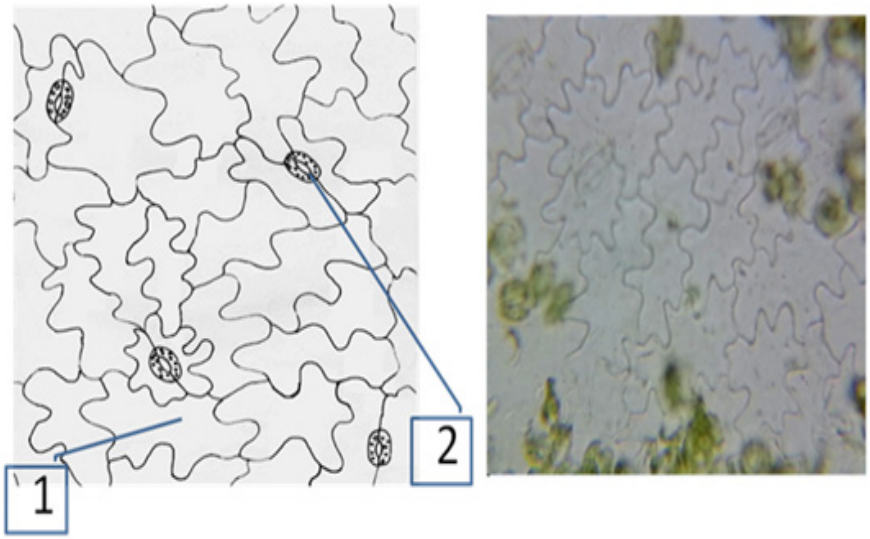

Figure 6: The structure of the upper epidermis of *Vicia truncatula Fish. ex Bieb*. leaf plate

consists of an investing tissue, mesophylland conducting bundle. The investing tissue and epidermis are presented by the epidermis cells. Mesophyll is presented by a palisade type and a spongy type. The mesophyll palisade settles on two layers under the top layer of epidermis. The spongy mesophyll settles on two layers between the mesophyll palisade and alow top of epidermis. The spongy mesophyll is presented by live parenchyma cells. The carrying-out system has aclustered type. (Figure 4)

The structure of bottom epidermis of leaf plate

The main cells of bottom epidermis have strongly twisting anticlinal walls. The stomatal apparatus belongs to anomocytic type. It has thick covering by simple multicellular hairs. (Figure 5)

The structure of upper epidermis of leaf plate

The epidermis main cells represent wavy anticlinal walls. The stomatal apparatus belongs to anomocytic and paracytic types. (Figure 6)

The cross cut of a petiole of a leaf

The petiole has a saddle-type shape on the top and triangularshape on the bottom. There is an epidermis on the outside which consists of unicellular hairs. There is a collenchyme under the epidermis which consists of several layers. Chlorenchyme is disposed father. It consists of small cells withathin cellular wall withchloroplasts. There are five open collateral carrying-out bunches inside the petiole. Bunches are presented by sclerenchyme, phloem,

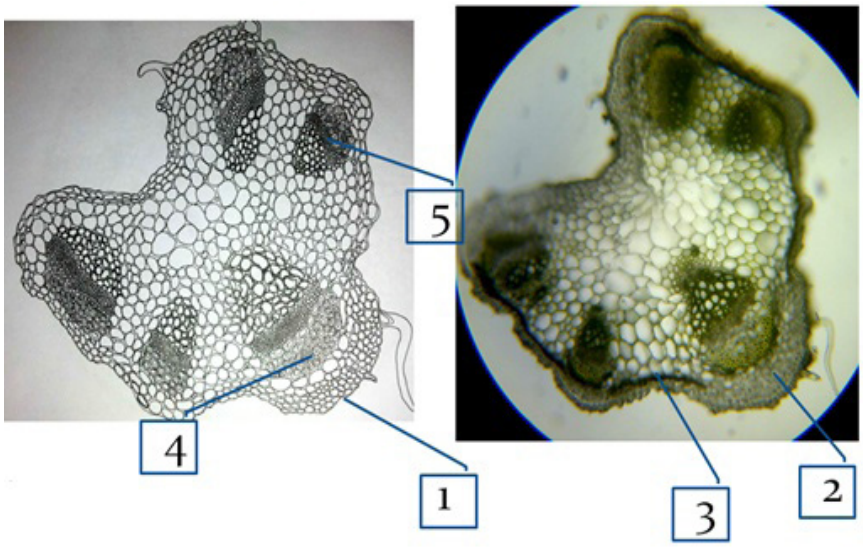

Figure 7: The anatomic structure of *Vicia truncatula Fish. ex Bieb*. leaf petiole

cambium, xylem. (Figure 7)

DISCUSSION

Morphological, micromorphological and anatomical characteristics of *Vicia truncatula Bieb*. were examined in this study. There are a few information about morphological properties of in the Flora of USSR and Vascular Plants of Russia and Adjacent States. Results of comparison of morphological properties both genus are presented.

CONCLUSION

In conclusion we would like to note that these investigations are one of the phases of *Vicia truncatula Fish. ex Bieb*. examination and the medicinal plant is considered to be a perspective source of flavicine. The morphological and anatomical research obtained data can be used in standard documentation for medicinal vegetable raw materialcompiling.

CONFLICT OF INTEREST

When performing this research conflicts of interest did not arise, since all of the authors have conducted research work comprehensively.

ACKNOWLEDGEMENTS

This research project was carried out with the assistance of the Fund for the development of research "CLEVER". The authors are grateful to the head of Pyatigorsk medical and pharmaceutical Institute V. L. Adgienko.

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