Pharmacognostic and Preliminary Phytochemical Evaluation of the leaves of *Bixa orellana*

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Abstract

Various traditional systems of medicine enlightened the importance of the leaves of *Bixa orellana* (Bixaceae) to have a great medicinal value. The present study was aimed at pharmacognostic and preliminary phytochemical evaluations of *B. orellana leaves*. The pharmacognostic investigations were carried out in terms of organoleptic, microscopic and physical parameters. The dried leaves were subjected to successive Soxhlet extraction using petroleum ether, chloroform, ethyl acetate, methanol and water. These solvent extracts were subjected to a preliminary phytochemical screening to detect the different chemical principles present viz., carbohydrates, proteins, amino acids, steroids, glycosides, alkaloids, tannins and phenolic compounds. The phytochemical evaluation revealed the presence of carbohydrates, steroids, alkaloids, proteins, flavonoids, terpenoids, phenolics, tannins and glycosides.

Keywords: *Bixa orellana*; Pharmacognosy; Phytochemistry.

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INTRODUCTION

Chemicals derived from plants represent a plethora of drugs used today. These plant drugs also constitute a potential for developing some novel semisynthetic therapeutic agents. Over the last decade there has been a growing interest in drugs of plant origin and among the known plant species, only a small percentage has been investigated for phytochemicals and pharmacological activities. *Bixa* belongs to family Bixaceae and is represented by a single species *Bixa orellana*. One of the plants having many medicinal uses in traditional system of medicine is *Bixa orellana*. It is a shrub or bushy tree which ranges from 3 to 10 meters in height (15). Both roots and leaves are used for treating sore throats, jaundice, dysentery, gonorrhea, liver diseases and as antipyretic agent (4). *Bixa* is used in traditional Filipino cooking as a coloring agent. It is also used in coloring butter, margarine, cheese, beverages, meat and fish products. The *Bixa* dye also known as annatto dye is extracted from the outer covering of the seeds of *B. orellana* (2).

The leaves are used to treat skin problems, liver diseases and hepatitis. The decoction of leaves is used as a gargle for sore throat. An infusion is given in jaundice and dysentery. The leaves are also a popular febrifuge and are reported to possess anti tumor properties. The poultice of leaves is applied to cuts and gashes as a scar preventive (16). Today in Brazilian herbal medicine, a leaf decoction of annatto is used to treat heartburn and stomach distress caused by spicy foods and as a mild diuretic and mild laxative. It is also used for fevers and malaria and topically, to treat burns. Eight to ten dried leaves are boiled for 10 minutes in 1 liter of water for this popular Peruvian remedy. One cup is drunken warm or cold 3times daily after meals to treat prostate disorders and internal inflammation, arterial hypertension, high cholesterol cystitis, obesity, renal insufficiency, and to eliminate uric acid (13). This decoction is also recommended as a vaginal antiseptic and wound healer, as a wash for skin infections and for liver and stomach disorders. Curanderos (herbal healers) in the Peruvian Amazon squeeze the juice from the fresh leaves and place it in the eye for inflammation and eye infections, and they use the juice of 12 fruits taken twice daily for 5days to treat “epilepsy” (9).

The present investigation dealt with the pharmacognostic parameters of the leaves of *B. orellana*, and also with preliminary phytochemical evaluation of different solvent extracts of leaves. The leaves were evaluated to observe their organoleptic, microscopic

and physical parameters. The successive petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts of leaf powder of the plant were examined for its phytochemical principles.

MATERIALS AND METHODS

Chemicals

All the chemicals were of highest available purity and were procured from E. Merck, Mumbai, India, HiMedia Laboratories, Mumbai, India and SD Fine Chemicals, Mumbai, India.

Procurement of plant material

The leaves of *B. orellana* were collected from the wild growing tree in the Botanical Garden, Biotechnology Department in Kakatiya University, Warangal, India. Identification and confirmation was performed a qualified taxonomist. A specimen was deposited in the institutional herbarium. The collected plant material was made thoroughly free from any foreign organic matter. Leaves were separated, shade dried and powdered with laboratory mixer and sieved. Pharmacognostic studies were conducted with fresh leaves and leaf powder.

Pharmacognostic Evaluation

Organoleptic evaluation

In organoleptic evaluation, various sensory parameters of the plant material, such as size, shape, color, odor, and taste of the leaves were recorded (5). It includes conclusions drawn from studies resulted due to impressions on organs of senses.

Microscopic evaluation

The microscopic evaluation is essential for powdered crude drugs. Powders of the crude drugs consist of the fragments of cells in the form of recognizable tissues. Another important aspect of microscopic evaluation is the study of surface constants. The leaf constants like stomatal number, stomatal index, and palisade ratio were studied by using camera lucida. These constants are of diagnostic significance and are used for the authentication of leaf drugs or for the detection of their adulterants. Various diagnostic characters of leaves and leaf powder of *B. orellana* were studied by microscopic analyses with or without staining.

1. Powder analysis of leaf

To a little quantity of powder taken onto a microscopic slide, 1–2 drops of 0.1% phloroglucinol solution and a drop of concentrated hydrochloric acid were added, mounted in glycerol, covered with a cover slip and observed under microscope with 10 × 10 magnification. The characteristic features of the powder viz., vascular tissues, xylem fibers, calcium oxalate crystals, starch grains, trichomes etc. were recorded using standard techniques (1, 6, 8, 10, 11). Lignified cells, fibers and stone cells appear pink in color. Presence of starch grains was detected by the formation of blue color on addition of 2–3 drops of 0.01M iodine solution.

2. Determination of stomatal index

Leaf fragments of about 5 × 5 mm in size were taken in a test tube containing 5 ml of chloral hydrate solution and boiled on water bath until the fragments became clear (~15 min). These fragments were transferred onto microscopic slide, mounted in glycerol and observed under microscope for the presence and quantification of epidermal cells, stomata (type and distribution), palisade cells, vein islet number and veinlet termination number. The slide was examined with 40 × objective and 6 × eye piece to which a *camera lucida* was attached and recorded the epidermal cells and stomata lying within a selected area. Stomatal index was calculated as the percentage of number of stomata present per number of epidermal cells and each stoma was counted as one cell.

Physical evaluation

In physical evaluation, crude fiber, moisture content, ash values viz., total ash, acid insoluble ash and water soluble ash, and extractive values viz., alcohol soluble extractive value, water soluble extractive and ether soluble extractive values were determined (1, 6, 8, 10, 11, 17). The ash values represent the inorganic salts present in the drug. Extracts obtained by exhausting crude drugs are indicative of approximate measures of certain chemical compounds they contain, the diversity in chemical nature and properties of contents of drug. The determinations were performed in triplicate and the results are expressed as mean ± SD. The percentage w/w values were calculated with reference to the air-dried drug.

1. Estimation of crude fiber (Acid detergent fiber, ADF)

The ADF of the leaf composed of cellulose, lignified nitrogen, and alkali-soluble lignin. In a 500 ml Berzelius beaker, 2 g of leaf material was distilled by refluxing with 50 ml ADS (20gm cetyl trimethyl ammonium bromide (cetrimide) in 1 litre of previously standardized N sulphuric acid) by boiling vigorously at first and then more gently. After 1 hr of reflux distillation, the contents

of the beaker were transferred to a tared crucible and the contents were allowed to percolate through the sintered glass plates. The residue was repeatedly washed with boiling water until no more foam appeared in the filtered solution. The residue was sucked dry and washed with 3 × 20 ml of acetone and finally sucked dry. The crucibles were kept overnight in a hot air oven at 100 °C, cooled in a desiccator and weighed. The residue which remained insoluble in the hot ADS was the amount of ADF in given sample. The ADF content of leaf powder was calculated.

2. Moisture content (loss on drying)

Ten gram of accurately weighed fresh leaves of *B. orellana* was placed in a tared evaporating dish and dried at 105 °C for 5hrs and weighed. Drying and weighing was continued at one hour interval until difference between two successive weighings corresponded to not more than 0.25%. Constant weight was reached when the difference in weight of two consecutive weighings was not more than 0.01gm after drying for 30 minutes and cooling for 30 minutes in desiccator.

3. Determination of total ash

Two gram of leaf powder of *B. orellana* was taken in a tared silica crucible and incinerated at a temperature not exceeding 450 °C until free from carbon. The resultant ash was cooled and weighed. The percentage of ash was calculated with reference to the air-dried drug.

4. Acid-insoluble ash

The total ash obtained from 2g of leaf powder was boiled for 5 minutes with 25 ml of dilute hydrochloric acid and the insoluble matter was collected on an ashless filter paper. It was washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

5. Water soluble ash

The total ash obtained from 2g of leaf powder was boiled for 5 minutes with 25 ml of water and the insoluble matter was collected on an ashless filter paper. It was washed with hot water, ignited and weighed. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

6. Determination of alcohol soluble extractive

Accurately weighed powder (5 g) of leaves was taken and macerated with 100 ml of 95% alcohol for 24 h. The contents were frequently shaken during the first 6 h and allowed to remain for 18 h. After 24 h, the extract was filtered and 25 ml of the filtrate was evaporated. The extract was dried at 105°C to a constant weight.

7. Determination of water soluble extractive

Water soluble extractive value was determined using the procedure described for alcohol soluble extractive, except that chloroform water was used for maceration.

8. Determination of ether soluble extractive

Accurately weighed powder (5 g) of leaves was taken and a thimble pack was prepared. The crude drug in the pack was extracted with solvent ether in a continuous extraction (Soxhlet) apparatus for 6 h. The extract was filtered; the filtrate was evaporated and dried at 105°C to a constant weight.

Preliminary Phytochemical Screening

The leaf powder was subjected to successive extraction in a Soxhlet apparatus using petroleum ether (60–80°C), chloroform, ethyl acetate, methanol and water for 8 hours and the extracts were evaporated to dryness. The dried extracts were weighed, and percentage yields were calculated. The extracts were used for preliminary phytochemical screening with a battery of chemical tests viz., Molisch's, Fehling's, Benedict's and Barfoed's tests for carbohydrates; Biuret and Millon's tests for proteins; Ninhydrin's test for amino acids; Salkowski and Liebermann-Burchard's reactions for steroids; Borntrager's test for anthraquinone glycosides; foam test for saponin glycosides; Shinoda and alkaline tests for flavonoid glycosides; Dragendorff's, Mayer's, Hager's and Wagner's tests for alkaloids; and ferric chloride, lead acetate, potassium dichromate and dilute iodine tests for tannins and phenolics (3, 7, 12, 14).

RESULTS AND DISCUSSION

Pharmacognostic Evaluation

Organoleptic and microscopic evaluation

In organoleptic evaluation, appropriate parameters like taste, odor, size, shape and color of the leaves and leaf powder were studied. Macroscopically, the leaf is simple in composition, opposite in arrangement, apex and base are acute, margin is entire, and average leaf size is 7.5 ± 0.9 cm (length) and 4.6 ± 0.2 cm (breadth). Fresh leaves are green in colour and characteristic in odour with a slightly bitter taste. The leaf powder was also green in colour with characteristic odour and bitter taste.

Micromorphological features revealed that the leaf powder contains numerous raphide shaped calcium oxalate crystals and starch grains, both simple and compound. The powder also showed the presence of xylem and phloem. Multicellular, long and covering trichomes seen were lignified.

The quantitative microscopic evaluation of fresh leaves and leaf powder was performed and the results obtained were shown in table 1 and figures 1, 2 and 3. The lower epidermal layers of fresh leaves have shown the presence of stomata with one or more subsidiary cells parallel to the long axis of the pore and guard cells which indicates paracytic arrangement. Upper epidermal layers were devoid of stomata.

Physical evaluation

The various physical parameters of leaves and leaf powder viz., crude fiber (acid detergent fiber), moisture content, ash values viz., total ash, acid insoluble ash and water soluble ash, and extractive values viz., alcohol soluble extractive value, water soluble extractive and ether soluble extractive values were determined. The results of this study were shown in table 2.

The results of these investigations could serve as a basis for proper identification, collection and investigation of the plant. The macro and micro morphological features

Table 1. Quantitative microscopy of leaf / leaf powder of *Bixa orellana*

PARAMETER	VALUE
Phloem fibers (width)	10.8 – 72µ
Starch grains (diameter)	13.32– 33.3µ
Calcium oxalate crystals (length)	26.64– 66.6µ
Stomatal number (lower epidermis)	275 / mm^2
Stomatal index (lower epidermis)	4.13
Vein islet number	24/ mm^2
Veinlet termination number	11 / mm^2
Palisade ratio	6.25 / mm^2

Table 2. Physicochemical parameters of leaf powder of *Bixa orellana*.

PARAMETER	VALUE % w/w
Acid detergent fiber	28.5
Moisture content	60.7
Total ash	5.66
Acid insoluble ash	0.66
Water soluble ash	2.33
Alcohol soluble extractive value	8.4
Water soluble extractive value	12.2
Ether soluble extractive value	8.57

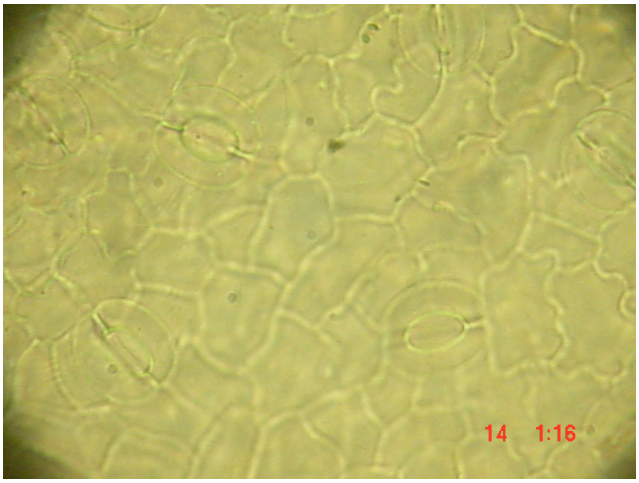

Figure 1. Stomata in lower epidermis of leaf

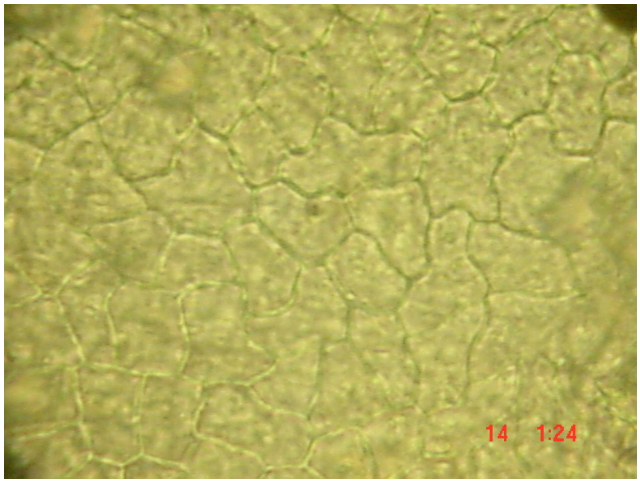

Figure 2. Stomata in upper epidermis of leaf

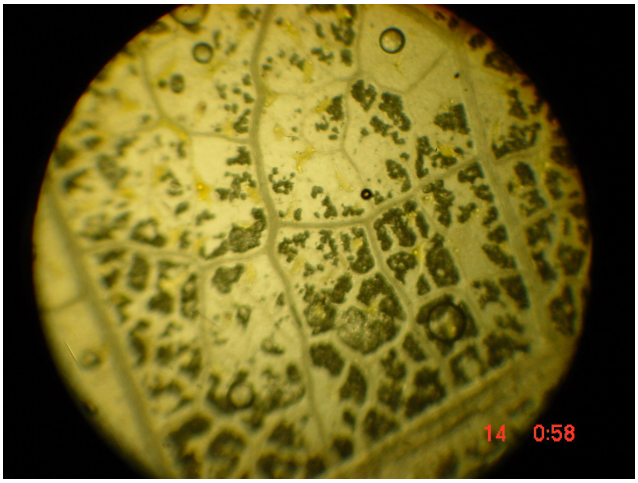

Figure 3. Vein islets in lower epidermis of leaf

of the leaf described, distinguishes it from other members of the genera. Numerical data and quantitative leaf microscopy are parameters that are unique to the plant and are required in its standardization.

Preliminary Phytochemical Evaluation

The leaf powder of *B. orellana* was extracted with petroleum ether, chloroform, ethyl acetate, methanol and water and the nature and yield of the extracts were observed. Petroleum ether, chloroform and methanol produced resinous extracts whereas ethyl acetate and aqueous extracts were solid in nature. All the solvents produced dark green coloured extracts from the leaf powder. Among the solvents employed chloroform produced highest yield with lowest yield extracted out by water. The yields of the extracts were found to be 8.57, 22.65, 2.66, 12.4 and 1.12 % w/w respectively.

The petroleum ether and chloroform extracts of the powdered leaves of *Bixa orellana* showed the presence of steroids and terpenoids. The ethyl acetate extract responded positively to the tests for steroids, terpenoids, phenolics and tannins. Methanolic extract of the leaves produced positive tests for flavonoids, steroids, terpenoids, phenolics, tannins, alkaloids and glycosides. Aqueous extract showed the presence of carbohydrates, proteins, flavonoids, phenolics, tannins and glycosides. These secondary plant metabolites are known to possess various pharmacological effects and might be responsible for the various actions exerted by *B. orellana*.

B. orellana is used in the treatment of various disease conditions. The standardization of a crude drug is an integral part of establishing its correct identity. Before any crude drug can be included in a herbal pharmacopoeia, pharmacognostic parameters and standards must be established. The results of the present investigations could serve as a basis for proper identification, collection and investigation of the plant. The macro-and micro-morphological features of the leaf described, distinguishes it from other members of the genera. Numerical data and quantitative leaf microscopy are unique to the plant and are required in its standardization. Phytochemical evaluation revealed the presence of various secondary plant metabolites which have been claimed to be responsible for various pharmacological activities.

CONCLUSION

The pharmacognostic parameters, which are being reported for the first time, could be useful in the identification and standardization of a crude drug. The data produced in the present investigation is also helpful in the preparation of the crude drug's monograph and inclusion in various pharmacopoeias.

REFERENCES

1. Ansari S.H., *Essentials of Pharmacognosy*, (Birla Publications, Delhi, 2005–06) 207–594.
2. Aranez A.T. and Rubio R.O.. Genotoxicity of pigments from seeds of Bixa orellana L. (Atsuete) I. Determined by allium test. *Philippine J Sci.* **125**: 259–269 (1996).
3. Bruneton J., *Pharmacognosy, Phytochemistry, Medicinal Plants*, (Intercept, Hampshire, 1999) 131–160.
4. Castello M.C., Phatak A., Chandra N. and Sharon M.. Antimicrobial activity of crude extracts from plant parts and corresponding calli of *Bixa orellana*. *Indian J Exp Biol*. **40**: 1378–1381 (2002).
5. Evans W.C., *Trease and Evans Pharmacognosy*, (WB Saunders Ltd, London, 2002) 32, 33, 95–99, 512, 547.
6. Goyal R.K. and Shah B.S., *Practicals in Pharmacognosy*, (Nirali Prakashan, Pune, 2005) 128–155.
7. Harborne J.B., *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, (Springer Verlag, Berlin, 2005) 107–200.
8. Heinrich M. and Barnes J., 2004, *Fundamentals of Pharmacognosy and Phytotherapy*, (Churchill Livingstone, Pune, 2004) 24–29.
9. Joshi S.G., *Medicinal plants*, (Oxford and IBH publishing Co Pvt. Ltd, New Delhi, 2000) 99.
10. Khandelwal K.R., *Practical Pharmacognosy*, (Nirali Prakashan, Pune, 2003) 38–161.
11. Kokate C.K., *Practical Pharmacognosy*, (Vallabh Prakashan, Pune, 2002) 107–129
12. Mukherjee P.K., *Quality Control of Herbal Drugs*. *An Approach to Evaluation of Botanicals*, (Business Horizons Pharmaceutical Publications, New Delhi, 2002) 529–533.
13. Parotta J.A., *Healing plants of peninsular India*, (CABI Publishing, London, 2001) 176–177.
14. Rangari V.D., *Pharmacognosy and Phytochemistry*, (Career publications, Nashik, 2004) 129–150.
15. Srivastava A., Shukla Y.N.M., Jain S.P. and Kumar S., Chemistry, pharmacology and uses of *Bixa orellana* – a review. *J Med Aromat Plant Sci.* **21**: 1145–1154 (1999).
16. Anonymous, *The Wealth of India (Raw Material)*, Vol-**2B** (Council of Scientific and Industrial Research, New Delhi, 1998) 157–160.
17. Alabi D.A. and Alausa A.A., Evaluation of the mineral nutrients and organic food contents of the seeds of *Lablab purpurus, Laucaena leucocephala* and *Mucua utilis* for domestic consumption and industrial utilization, *World J Agric Sci.* **2**: 115–118 (2006).

Micromorphological Studies on *Gmelina arborea* and *Clerodendrum serratum*

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Abstract

The crude root drugs of *Gmelina arborea* Roxb. and *Clerodendrum serratum* Moon are often adulterated with roots of *Gmelina asiatica* Linn. and *Premna herbacea* Roxb. respectively. The present paper provides certain anatomical standards for diagnosing the roots of the afore mentioned original drugs from their adulterants.

Keywords: *Gmelina arborea*, *Gmelina asiatica*, *Clerodendrum serratum*, *Premna herbacea*, Micromorphology..

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INTRODUCTION

The hypogeal organs of plants serve as the sink for many metabolites. The storage roots and rhizomes which are variously modified in to different exomorphic forms are primarily endowed with storage function. These storage products have proved to be of high economic value, especially in terms of medicinal properties (1-2). When the underground organ excavated from the ground, there are always chances for accidental mixing of roots of other growing in the population. Surface morphology of the roots, of course, may help largely to diagnose the roots in question. However, the external feature will be of limited reliability in certain cases where the original specimens and the adulterants simulate very closely. Under such predicament, analyses of internal structure of the root have to be of dependable resort.

In the present investigation some renowned root-drug used in indigenous formulations namely, *Gmelina arborea* Roxb. (Fig. 1-2), *Clerodendrum serratum* Moon, *G.asiatica* Linn. and *Premna herbacea* Roxb. (syn. *Pygmaeopremna herbacea* Moldenke) have been studied anatomically and the data are presented for standardizing the identity of the roots. It has been noted that the pioneer work has been conducted on the study of external features of the herb and their officinal part (*G.arborea* and *G.asiatica*) (3). Not much work has been done on the anatomical structure, especially on the root woods. The root woods of the plants have been given more accents in the present study, since the drugs available in the market include the wood as well as bark.

MATERIALS AND METHODS

Authentic root wood pieces of all four species were collected from the field after establishing their taxonomic identity. Thick root woods were cut in to small pieces and boiled in water till the specimens started sinking. The specimens were pickled in glycerin alcohol mixture for further softening. Sections of 15-20µm thickness in transverse and longitudinal planes were taken with sledge microtome. The lateral roots, less then 5mm thick, were embedded in paraffin wax after dehydration and wax infiltration and sectioned with rotary microtome to the thickness of 10µm (4). Sections were stained with toluidine blue (5) or safranin. For the study of individual wood elements, wood tissues were macerated employing Jeffrey's fluid (6). Description of the wood structure is followed as per the terminology recommended by IAWA (7). Photomicrographs were taken with Nikon Alphaphot microscope unit.

OBSERVATION

Growth rings less distinct; diffuse porous; pores narrow, thick walled, circular or elliptic (Fig.2:2-3); 36.8-55µm in diameter; tyloses absent; pores of the central parts of secondary xylem are filled with amorphous gummy substance. Axial parenchyma apotracheal, diffuse or paratracheal and scanty; occurs in vertical strands. Xylem rays predominantly biseriate, the two ends have very long, uniseriate, vertical row of tails (Fig.2:4); height of the rays 230-690µm; breadth 27.6µm. Imperforate tracheary elements include thick walled, narrow lumened libriform fibres and fibre-tracheids. Libriform fibres with wide lumen are also frequent. In macerated preparations, the vessel elements present a high degree of variation. Some are cylindrical, narrow with very long tails at both or one end; very narrow, long pitted elements with sub-terminal perforations are very common; these elements simulate the fibres (fibriform-vessel elements) in general shapes and size (Fig.2:5-6).

Growth rings fairly distinct, diffuse porous, growth rings demarcated by a broad zone of tangentially oblong, thick walled, narrow lumened fibres (Fig.3:2); pores

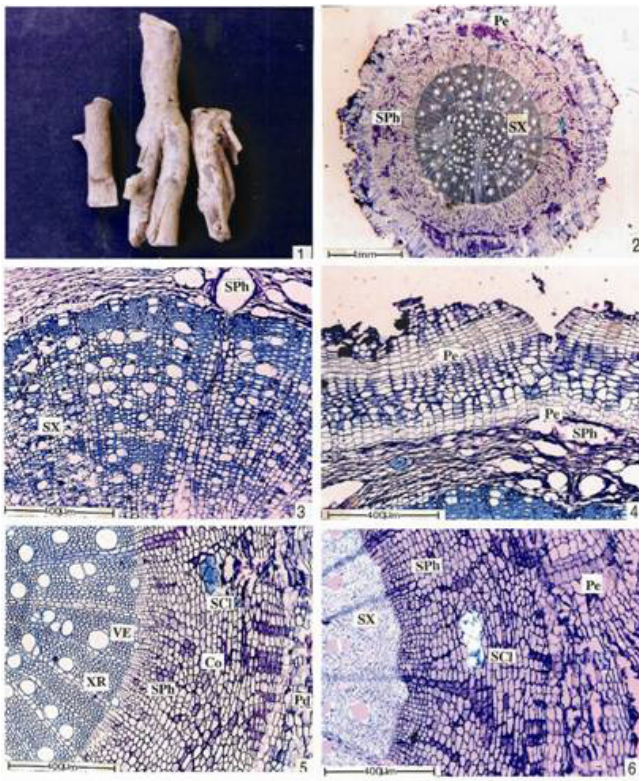

Figure – 1. *Gemilna arborea* Roxb.
1. Pieces of root drug showing external features
2. CS of thin lateral root.
3. secondary xylem of lateral root
4. Two zones of periderm of the lateral root
5. Secondary phloem and cortical tissues of thin lateral root
6. CS of lateral root under polarized light

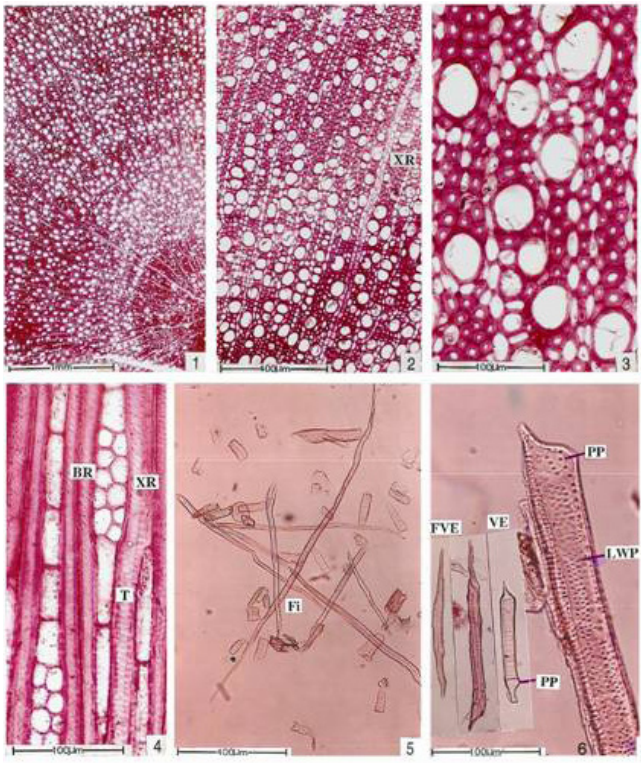

Figure – 2. *Gemilna arborea* Roxb.
1. CS features of old root
2. Secondary xylem showing growth rings
3. secondary xylem elements as seen in cross section
4. TLS of root wood showing biseriate ray with very long tails
5. Macerated different forms of vessel elements and cell wall pits

predominantly in tangential chains or less frequently solitary; circular in cross-sectional outline (Fig.3:2-3); diameter of the pores 50-145 (93)µm; Vessel elements cylindrical, with or without tails; lateral wall pits with angular borders and elliptical aperture; perforation plate simple, horizontal vessels filled with lignified tyloses forming pseudoparenchymatous structures within the vessel (Fig.3:3-4). Axial parenchyma paratracheal, vasicentric forming a thin, less conspicuous sheath around the vessels. Xylem rays 1-3 seriate, homo or heterocellular; rays 2-many cells in height, measuring 165-506 (400)µm high and 40-46µm in breadth; fibres libriform type, thick walled, lumen fairly wide, pits well developed on the radial walls.

Growth rings quite distinct, diffuse porous, growth rings demarcated by thick walled, narrow lumened fibres; pores circular, thin walled; diameter of the pores 37.4 – 188 (102) µm; pores mostly solitary, less frequently in radial multiples (Fig. 4:3-4); individual vessel elements cylindrical, short, with simple horizontal perforation plate; intervascular pits bordered, borders angular, aperture elliptic; scalariform pits are also frequent,

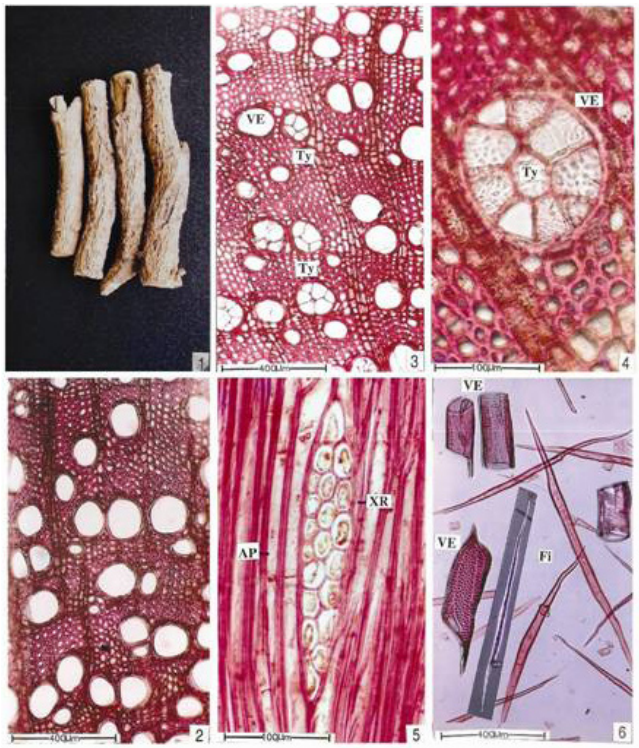

Figure – 3. *Gemilna asiatica* Linn.
1. External features of the root
2. CS of root showing vessel element cross sectional features
3, 4. Vessels with tyloses
5. TLS of root wood showing xylem rays
6. Macerated vessel elements and fibres

Figure – 4. *Clerodenrum serratum* Moon
1. Pieces of root drug
2. Gross anatomical features of root wood in cross section
3. A growth ring zone enlarged
4. CS of root wood showing vessel distribution with a growth ring
5. CS of root showing minute crystals in the xylem ray under polarized light

especially in the long narrow vessel elements (Fig.5). Axial parenchyma not abundant; either apotracheal or paratracheal and scanty. Xylem rays 1-3 seriate, homo or heterocellular; height of the rays 276-1012 (546.6) µm, breadth 55.2-110 (88.4) µm, needle-like crystals are abundant in the ray cells. Imperforate tracheary elements include, thick walled, simple pitted libriform fibres (Fig.5).

The drug consists of nodulated root / rhizome. The structure of thin and nodulated part is basically similar. The narrow part of the root system has well developed periderm with deep irregular fissures. The first periderm is superficial in origin comprising 5-10 layers of homogenous phellem cells. The subsequent periderm originates deeper in the cortical zone or from the outer zone of the secondary phloem. These two periderm layers constitute what is known as rhytidome (Fig.6; 2, 6). The rhytidome is followed by a broad zone of secondary phloem where small nest of sclerenchyma elements are embedded. The secondary xylem forms a broad, dense cylinder with wide pith in the case of underground stem or without pith if it is root. Secondary xylem has distinct one or two growth zones. The elements of secondary xylem include narrow regular thin walled solitary vessels and thick walled libriform fibres. The pith when present is parenchymatous and compact.

The nodulated part of the organ may be either underground stem (rhizome) or root. In the case of rhizome there is distinct wide parenchymatous pith surrounded by several primary xylem strands (Fig. 6:3). The bulk of the organ consists of radial fibres of cells reaching upto the periphery. This zone has mostly radially oblong thin walled parenchymatous cells. However there are several circular narrow bands of tangentially oblong thick walled cells and isolated narrow indistinct vessels. These circular zones resemble somewhat the growth ring pattern of mature wood (Fig. 6: 4-5). All the cells of the radial fibres heavily loaded with starch grains (Fig. 6:7). The surface of the rhizome has broad secondary phloem with nests of sclerenchyma elements as in the non-nodulated bark. The rhytidome with two or three wavy periderm zones are well developed with fissures. This rhytidome portion is similar to that seen in the non-nodulated thin portion.

In the case of root organ, the central part is occupied by primary xylem elements without distinct pith

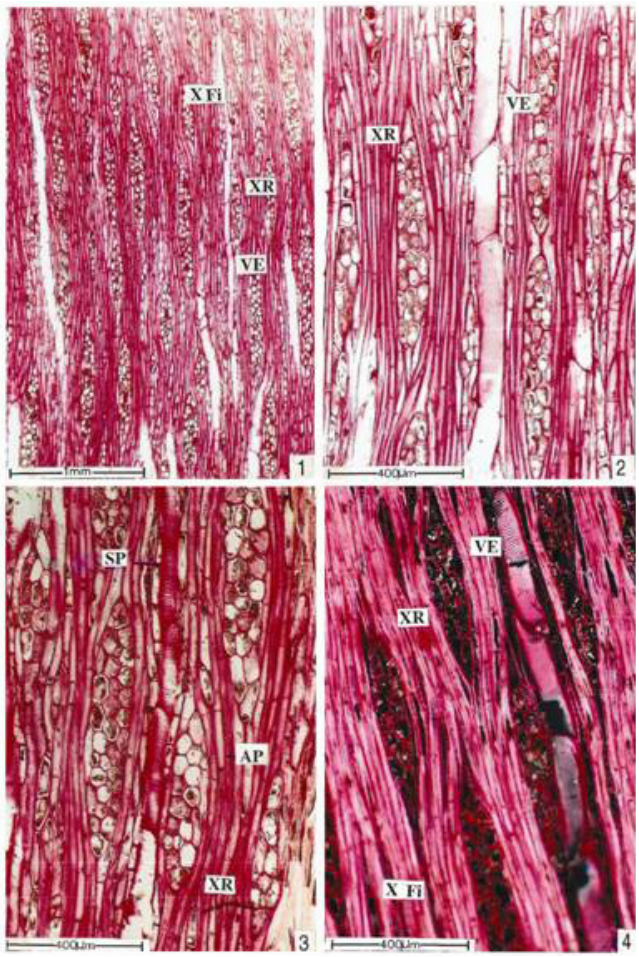

Figure – 5. *Clerodenrum serratum* Moon
TLS features of root wood

zone. The other features of the organ are similar to the rhizome. However, the outer zone of the tuberous root, the vascular elements exhibit twisting and curling in the form of irregular nodules. The structures are probably brought about by imbalanced proliferation of xylem parenchyma.

DISCUSSION

The root of *G.arborea* is used in the indigenous formulations as one of the important constituents, specifically as an ingredient of Dasamula. Because its restricted availability this drug is often adulterated with or substituted by easily available root drugs such as *G.asiatica*. In fragmentary form of small pieces of wood sold in the market it is difficult to distinguish *G.arborea* from its adulterants. However, the histological features of *G.arborea* root wood are specific. These features can be employed for distinguishing *G.arborea* from *G.asiatica*.

The growth rings in *G.asiatica* are fairly distinct with diffuse porous distribution of vessels (Fig. 3). In *G.arborea*, the growth rings are not evident. The pore diameter seems to be strikingly different in these two species (Fig. 1). In *G.asiatica* the mean pore diameter is 93 µm, whereas in *G.arborea* it is only 45 µm. The xylem rays are very unique in *G.arborea*. The rays are predominantly biseriate with very long tails. In *G.asiatica*, the rays are 1 to 3 seriate without tails. The vessels are often filled with abundant tyloses in the root wood of *G.asiatica*, whereas in *G.arbores* tyloses are not evident. Another striking difference may be noted in the macerated vessel elements of the two species. In *G.arborea*, fibriform – vessel elements are quite

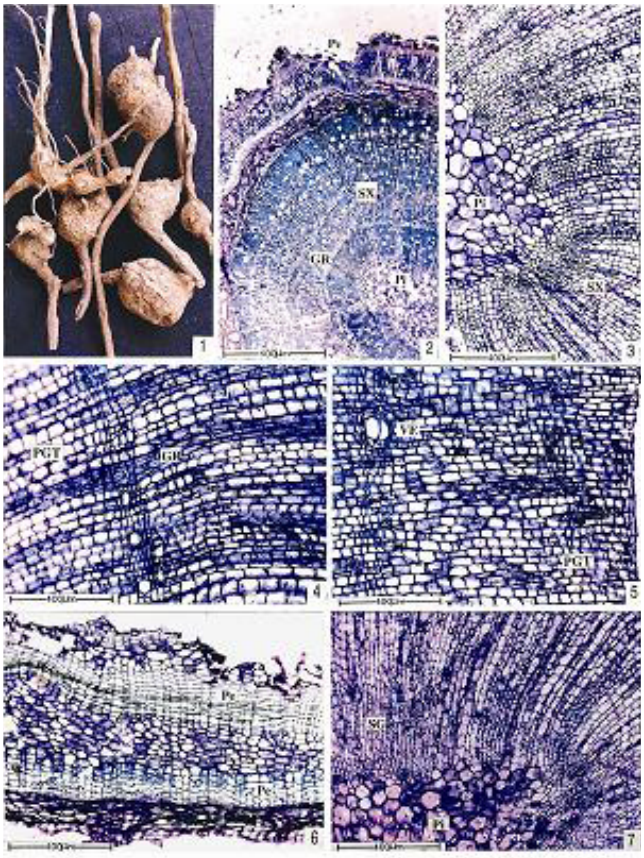

Figure – 6. *Premna herbacea* Roxb.
1. Root drug showing nodular swellings and narrow thin region
2. CS of thin portion of the drug
3. Inner portion of the nodular region showing pith and inner portion of the xylem cylinder
4. Middle part of the secondary xylem showing a narrow band of growth ring
5. Outer zone of the secondary xylem showing a growth ring with vessel elements and radial rows of parenchyma cells
6. Two successive zones of periderm
7. Pith and secondary xylem tissues have abundant starch grains as seen in the polarized light
(AP - Axial parenchyma; BR – Biseriate rays; Co – Cortex; Fi – Fibres; FVE – Fibriform vessel element; GR – Growth ring; LWE – Lateral wall pits; MPh – Medullary phloem; Pd – Phelloderm; Pe – Periderm; PGT – Parenchymatous tissue; PP – Perforation plate; Scl – Sclereids; SG – Starch grains; SP – Scalariform pits; SPh – Seconadary phloem; SX – Secondary xylem; T – Tail; Ty – Tyloses; VE – Vessel element; XFi – Xylem fibres; XR – Xylem rays)

abundant. These elements are narrow and fibre-like and possess well developed pits and sub-terminal perforations. In *G.asiatica*, the vessels are typically in a tangential line which is a rare or unique feature. In *G.arborea* the distribution of vessels is random and diffuse.

Two different drugs are sold in the market in the name of Bharangi. One is equated to *Clerodendrum serratum*. The other one is *Premna herbacea* (8). *Premna herbacea* has characteristic external morphology (Fig. 6) the roots are generally nodular with thin and spindle shaped thick portions along the length of the root. The thin portion of the root has dense secondary xylem cylinder with one or two growth rings. The periderm is composed of two or three zones of phellem alternating with non periderm tissues. It is typically of rhytidome type. The swollen nodular portion has somewhat anomalous structure. The secondary xylem is usually centric in growth and consists of mostly thin walled parenchyma cells arranged in radiating rows of cells. There are several growth ring-like narrow zones of thick walled cells and few narrow vessels. The nodular organ is primarily storage in function.

The roots of *Clerodendrum serratum* have typical dicotyledonous root structure (Fig. 4, 5). The root wood has distinct growth rings. The individual vessel elements bear either circular or scalariform bordered pits. The xylem rays are quite broad and possess needle like calcium crystals. The above mentioned characters may be largely aid in distinguishing the root drugs in fragmentary form.

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REFERENCES:

1. Chopra RN, Nayar SL, Chopra IC. *Glossary of Indian Medicinal Plants*, CSIR, New Delhi, Publication & Information Directorate, 1956.
2. Nadkarni AK. *Indian Materia Medica*, Mumbai, Popular Prakashan Pvt. Ltd; 1982.
3. Narayana Iyer K, Kolammal M. *Pharmacognosy of Ayurvedic drugs*, Trivandrum, Kerala, Department of Pharmacognosy, University of Kerala; Series 1. Vol.**9**, 49–56, 1978.
4. Sass JE. *Elements of Botanical Microtechnique*, New York and London, McGraw Hill; 1940.
5. O'Brien TP, Feder N, McCull ME. Polychromatic staining of plant cell walls by toluidine blue *O, Protoplasma*. **59**: 364 – 373, 1964.
6. Johansen DA. *Plant Microtechnique*, New York, McGraw Hill; 1940.
7. IAWA. List of microscopic features for hardwood identification, *IAWA Bull*. **10**: 219 – 332, 1989.
8. Anonymous. *The Wealth of India - A Dictionary of Indian Raw Materials & Industrial Products*, CSIR, New Delhi, Publication & Information Directorate, Vol. **8** (Ph-Re), 1969.

Standardisation of *Artemisia annua* using Reversed Phase High Performance Liquid Chromatography (RP-HPLC).

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Abstract

Artemisia annua L is a traditional Chinese medicine for the treatment of many disorders including drug resistant malaria. The active constituent against malaria, artemisinin, has been isolated and characterized by Chinese scientists. *Artemisia annua* is now being grown in malaria endemic countries like Nigeria. The aim of the present study was to quantify artemisinin content of Nigerian Grown *Artemisia annua* using a fast and reliable Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) analytical technique since artemisinin content is affected by geographical and seasonal variations. HPLC conditions used for determination of the artemisinin content were established as follows; phenomenex luna column (5 µm; 250 mm × 4.6 mm) was employed with the mobile phase of acetic acid (% 0.1 v/v): acetonitrile: H_2O (70:30) mixture at the flow rate of 1 ml/min. The good linearity of artemisinin was observed with y = 11714 × (r^2 = 0.9989). Artemisinin was detected in our sample and was calculated to be 1.0975 %.

Keywords: *Artemisia annua* L., Artemisinin, HPLC, Standardisation

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INTRODUCTION

Approximately two billion people live in malaria endemic areas. The incidence of this disease is increasing dramatically because malaria parasite strains have become resistant to the available drugs. [1]

Artemisinin has been used in traditional Chinese herbal fever remedies for more than 1,500 years.[2] It is isolated from sweet wormwood (*Artemisia annua*), and its skeleton is used as the basis for the synthesis of several other modern antimalarial drugs, such as artesunate and artemether. These artemisinin derivatives are important in the treatment of drug-resistant malaria. They are presently the most potent antimalarials available, rapidly killing all blood stages of the malaria parasite *Plasmodium falciparum*.[3]

Nurgun, *et al*. 2007,[4] reported a number of analytical methods that have been developed for detection and quantification of artemisinin, e.g., thin layer chromatography (TLC), TLC with visible light densitometric detection, high-performance liquid chromatography with UV detection (HPLC–UV), HPLC with electrochemical detection (HPLC–ECD), HPLC with evaporative light scattering detection (HPLC–ELSD), HPLC with peroxyoxalate chemiluminescence detection (HPLC-PO-CL), HPLC/tandem mass spectrometric (LC/MS/MS) method, liquid chromatography/mass spectrometry (LC/MS), high performance capillary electrophoresis using self-designed conductivity detection system, gas chromatography with mass spectrometric detection (GC–MS), GC with flame ionization detection (GC–FID), enzyme-linked immunosorbant assay

(ELISA) and Reversed Phase High Performance Liquid Chromatography.

An international agreement that will give traditional medicines a foothold in health systems have been endorsed by the World Health Organization (WHO). Member states of the United Nations have been called to formulate policies to ensure the safe and effective use of traditional medicines. The current challenge of researchers around the world is the difficulty in standardizing the active ingredients as these are often present in plant extracts that have regional or seasonal variations. Because of this, drug regulatory agencies around the world have hesitated in approving their use.[5] There is need therefore to begin to standardize the active constituents in medicinal plants grown in different parts of the world.

Artemisinin levels vary in amounts in naturally growing *Artemisia annua*. Artemisinin level in the leaves and flowering parts of the plant is affected by growth conditions, seasonal and geographical variations as well as breeding. [6] *Artemisia annua* in natural habitats contain 0.06-0.5 % artemisinin. Breeding has however made it possible to achieve artemisinin content of up to 2 %.[7]

The demand for Artemisinin is presently very high, and this has made producers to push up the price.[8] This global demand has made different countries of the world like Nigeria, for example to begin to cultivate the plant in large quantity for the local production of artemisinin in order to make the artemisinin based *combination* therapy affordable. Although the synthesis of artemisinin has been achieved, the cultivation of the plant is considered more economical than the synthetic alternative.[9–10]

In this present study, we examined the level of artemisinin in *A. annua* grown in Nigeria. This attempt was to standardise Nigerian grown *A. annua* with respect to artemisinin using reversed phase-high performance liquid chromatography (RP-HPLC) analytical technique.

MATERIAL AND METHODS

Chemicals and reagents

Standard artemisinin was purchased from Sigma-Aldrich Co. (USA). All the solvents used were of HPLC analytical-grade.

Preparation of Standard Solutions

100 mg of standard artemisinin was placed in a measuring flask and solubilised with 95 % ethanol which was added to the 100 ml mark. Serial dilutions of the stock solution were prepared as follows: An aliquot (2 ml) of artemisinin solution was transferred into 100 ml measuring flask and 8 ml of 95 % ethanol was added. This was labeled as "Solution-1". Similarly, "Solution-2" was made with 5 ml of artemisinin solution and 5 ml of 95 % ethanol and "Solution-3" was prepared with 10 ml of artemisinin solution alone without ethanol. This was followed by the addition of 40 ml of 0.2 % NaOH solution to the three flasks respectively, and then, allowed to react at 50 °C for 30 min.

After that, 0.08 mol/L acetic acid solution was filled up to the mark. Three standard solutions were prepared and applied to HPLC.[4]

Sample preparation and calibration curves
Plant Materials

Artemisia annua plant was obtained from the Molecular Biosciences Ltd, Calabar Cross River State, Nigeria where the plant was cultivated in 2008. The plant was identified at the Department of Botany of the University of Calabar, Cross River State, Nigeria.

Extraction procedure

Five grams of *Artemisia annua* plant was weighed accurately in triplicates and macerated in 250 ml of *n*-hexane at room temperature for 2 days using a laboratory-scale shaker. The *n*-hexane phase was filtrated and evaporated under vacuum until dryness. The residue was dissolved again in 100 ml of *n*-hexane and the *n*-hexane phase was washed in a separatory funnel with 2 % NaOH solution to get rid of impurities soluble in NaOH. The alkali solution present in the lower layer was discarded and the upper layer solution was washed several times with distilled water until it was neutralized. This solution was evaporated to dryness under vacuum at 45 °C using a rotary evaporator.

The extract, obtained after distillation was dissolved in 250 ml of 95 % ethanol and then filtered into a measuring flask. Then, 10 ml of filtered liquor was transferred into a 100 ml measuring flask, and 40 ml of 0.2 % NaOH solution was added to the flask and allowed to react at 50 °C for 30 min after which 0.08 mol/L acetic acid solution was added to fill up to the mark. [4]

Chromatography Equipment

The analysis was carried out with an LC system consisting of an HPLC Waters series quaternary pump with degasser and a photodiode array detector. Samples were injected with an HPLC Waters Autosamplers with thermostatted column compartment on an a phenomenex column (5 µm; 250 mm × 4.6 mm), at 30 °C. The system was controlled and data analyses were performed with Waters Empower 2 software. All the calculations concerning

the quantitative analysis were performed with external standardization by measurement of peak areas.

Calibration Solutions

In order to establish the linear detection range for each compound, individual standard stock solutions was prepared in mobile phase in 100 ml-measuring flasks. Aliquots of these solutions were diluted and analyzed to determine method linearity. Calibration ranges for artemisinin 5 – 100 ppm were prepared. Triplicate 10 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area was plotted against the concentration to obtain the calibration graph. The five concentrations were subjected to regression analysis to calculate calibration equation.

Procedure for HPLC Analysis

A mobile phase consisting of acetic acid (% 0.1 v/v): acetonitrile: H_2O (70:30) by isocratic elution was chosen to achieve maximum separation and sensitivity. Flow rate was 1.0 ml/min. Column temperature was set at 30 °C. The samples were detected at 254 nm using photodiode array detector. Result of artemisinin level in *Artemisia* sample was expressed as the mean of three determinations.

Linearity

Equation of the regression line formula was y = 11714x; R^2 = 0.9989 for artemisinin.

RESULTS AND DISCUSSION

Analytical data were obtained when artemisinin standard concentrations of 5, 10, 20, 50 and 100 ppm were injected into the HPLC system. For each of the standard concentrations, triplicate injections were utilized. The mean peak areas for 5, 10, 20, 50 and 100 ppm were 30596, 109160, 242947.7, 583807 and 11772787 respectively Table 1.0. The peak areas were plotted against the concentrations to give the calibration curve in Figure 1.0. The linear regression equation was y=11714 and r^2 = 0.9989 for artemisinin. Excellent linearity was obtained for artemisinin between peak areas and concentrations. The standard curve was used to estimate the artemisinin content of *Artemisia annua* extract.

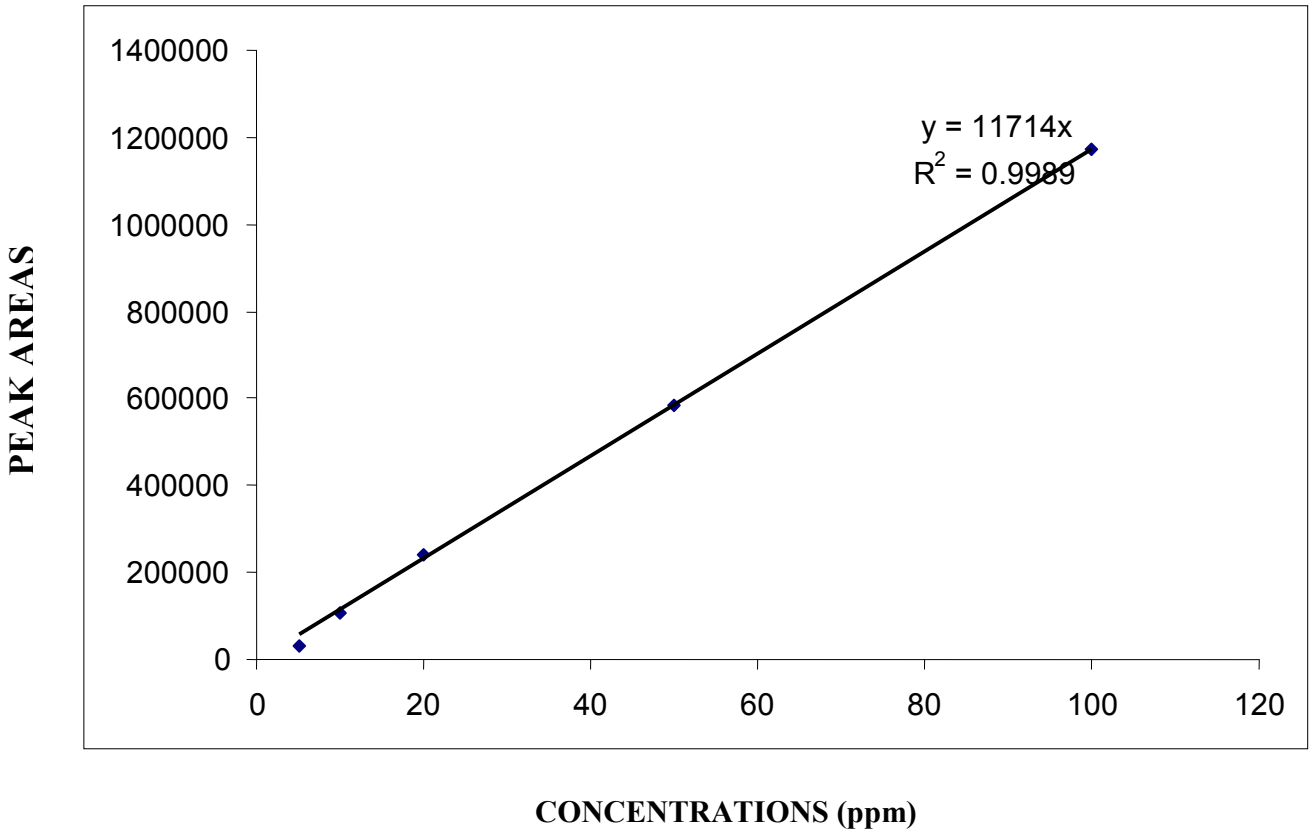


Figure 1: Calibration curve for the determination of artemisinin in Artemisia annua extract solution.

TABLE 1: ANALYSTICAL DATA OBTAINED FROM INJECTION OF ARTEMISININ STANDARDS UTILISED TO PLOT CALIBRATION CURVE

CONCENTRATIONS (ppm>	5	10	20	50	100
PEAK AREAS	30596	109160	243557	577306	1173799
PEAK AREAS	30596	109160	242643	586689	1172806
PEAK AREAS	30596	109160	242643	587426	1171757
PEAK AREAS	30596	109160	242947.7	583807	1172787

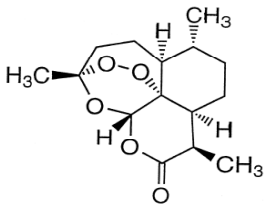
TABLE 2: PEAK AREAS OFARTEMISININ IN *ARTEMISLA ANNUA* EXTRACT

	ARTEMISIA EXTRACT 1	ARTEMISIA EXTRACT 2	ARTEMISIA EXTRACT 3
AREA 1	26350	25236	25578
AREA 2	24449	24512	23683
AREA 3	25409	24526	26337
AREA 4	27342	27368	27614
AREA 5	26837	27014	26737
AREA 6	26681	26142	26920
AREA 7	27931	26617	26841
AREA 8	27806	26762	27163
AREA 9	28737	26462	27973
MEAN	**26838**	**26071**	**26538.44444**

TABLE 3: DETERMINATION OF ARTEMISININ CONCENTRATION IN *ARTEMISIA ANNUA* USING THE CALIBRATION CURVE. (y=11714x; R^2=0.9989)

ARTEMISIA	MEANS OF PEAK	CONCENTRATION
EXTRACT 1	26838	2.29
EXTRACT 2	26071	2.23
EXTRACT 3	26538.44	2.27
MEAN		**2.26**

The peak areas shown in Table 2.0 are the areas under the elution profile of triplicate samples of *Artemisia annua* extract solutions 1, 2 and 3 injected nine times each into the HPLC system. The mean values of these areas were 26838, 26071 and 26538.4 respectively. The



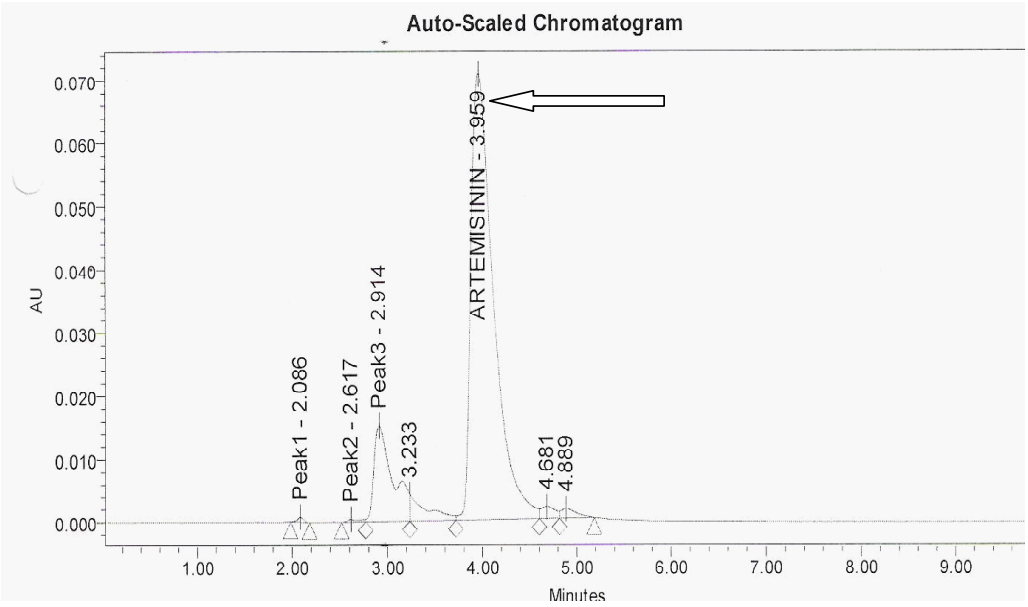


Figure 2: *Representative chromatogram of three determinations of standard solution of atremisinin* (RT=3.959). Peak area: 577306.

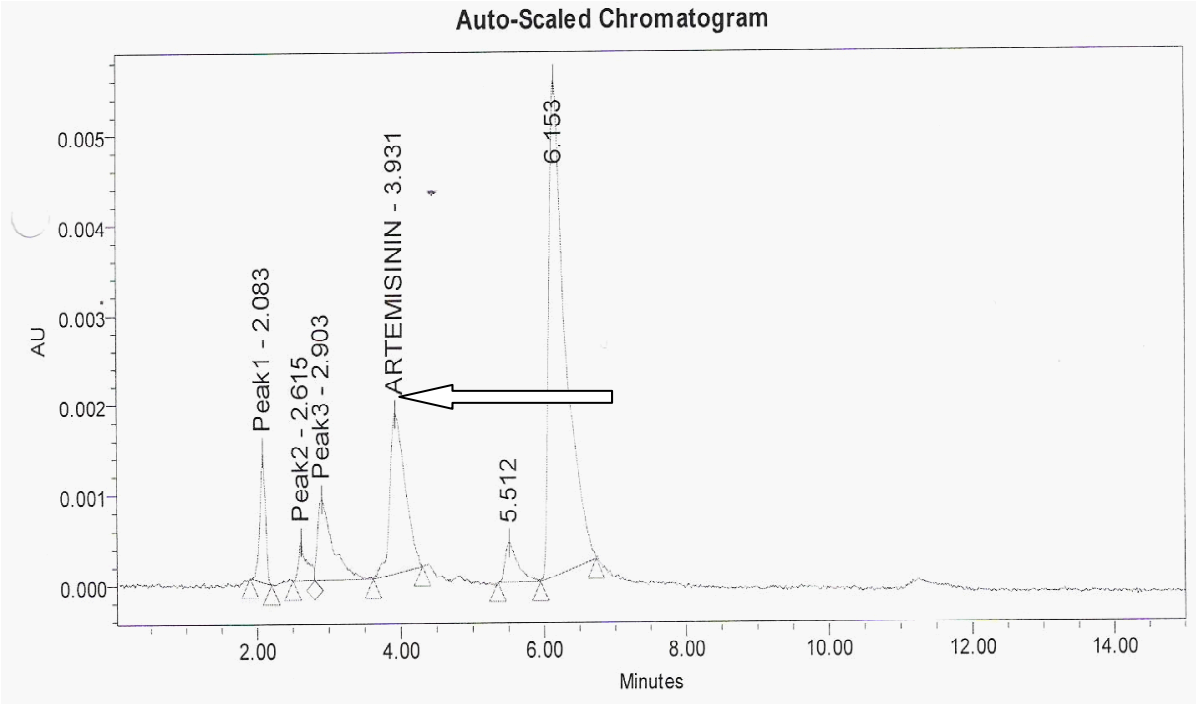


Figure 3: *Representative chromatogram of nine determinations of Artemisia annua triplicate extract solution* (RT=3.931). Peak area: 24526.

Table 4: PEAK AREAS AND CONCENTRATIONS DURING SPIKING

	***ARTEMISIA ANNUA* EXTRACT*+STANDARD ARTEMISININ**	***ARTEMISIA ANNUA* EXTRACT**+STANDARD ARTEMISININ**	***ARTEMISIA ANNUA* EXTRACT***+STANDARD ARTEMISININ**
EA1	79974	170766	350711
EA2	80701	169059	352537
EA3	78772	170766	352278
PEAK ***S ***ATIONS	79815.67	170197	351842
6.813698708	1452936657	30.03602527	

* 100µL of PLANT EXTRACT SPIKED WITH 300µl of 20ppm STANDARD ARTEMISININ.
** 100µL of PLANT EXTRACT SPIKED WITH 300µl of 50ppm STANDARD ARTEMISININ.
*** 100µL of PLANT EXTRACT SPIKED WITH 300µl of 1000ppm STANDARD ARTEMISININ.

corresponding concentrations were extrapolated from the calibration curve to give concentrations of 2.29, 2.23 and 2.27 respectively. The mean concentration of the triplicate samples of *Artemisia annua* extract solutions was 2.26 ppm (Table 3.0).

In order to be sure that the analysis was reproducible, accurate and reliable, recovery analysis was carried out using spiking procedure shown in Table 4.0 and recovery analysis data shown in Table 5.0. Recovery of extract 1, 2 and 3 were 99.1, 105.3 and 118.55 % respectively after spiking. Representative portions of the chromatograms of artemisinin standard and *Artemisia annua* extract solutions were as shown in Figures 2 and 3 respectively. The amount of artemisinin present in the Nigerian grown *Artemisia annua* was calculated to be 1.0975 %.

The search for artemisinin, the active antimalaria constituent of *Artemisia annua* is in progress. Synthetic alternative is not economical because it gives low yields. The only economical procedure is the cultivation of the plant. Since the discovery of the plant, different countries of the world have naturalized the plant. The aim is to make the plant available locally so as to drastically increase the amount of artemisinin used in the manufacture of artemisinin based combination therapy and to bring down the cost.[10–11] There is need to standardize the *Artemisia annua* cultivated in different parts of the world

Table 5: ANALYTICAL DATA OBTAINED FROM RECOVERY ANALYSIS

CONCENTRAT ION DETERMINED IN THE PLANT SAMPLE (ppm)	CONCENTRAT ION ADDED TO SAMPLE (SPIKING)(ppm)	EXPECTED CONCENTRAT ION (ppm)	OBTAINED CONCENTRAT ION (ppm)	RECOVERY (%)
2.26	4.615	6.875	6.814	99.10
2.26	11.538	13.798	14.529	105.30
2.26	23.076	25.336	30.036	118.55

because its levels is affected by the plant part collected, breeding methods, seasonal and geographical variations as well as growth conditions.[10] This standardization will give an idea of the amount of artemisinin to be expected before going on a large scale production. *A. annua* grown in the natural habitats contain 0.06–0.5 % artemisinin, but breeding yielded artemisinin content of 2 %. The levels also vary with geographical, seasonal as well as growth conditions as mentioned above. Artemisinin content of sixteen seed-generated lines of the cultivar *A. annua* ranged from 0.2 % to 0.9 % by both GC-FID and HPLC-ELSD.[12] HPLC analysis of artemisinin in *Artemisia annua* herb yielded 0.652 % by RH-HPLC method. [13]

Different solvents such dichloromethane, hexane, toluene, petroleum ether and chloroform can be used for the extraction of artemisinin from *Artemisia annua*. [11–15] In our study, n-Hexane was used because it is more selective in isolating artemisinin.[6] The calculated content of artemisinin in the Nigerian grown sample (1.0975 %) is comparable with the levels in other countries.

CONCLUSION

As Nigeria is embarking on a large scale production of the plant, the local production of artemisinin is a good development because the cost of production of artemisinin based combination therapy will be reduced compared with the past. The synthetic option is not presently feasible in Nigeria because it is not economical. The large vast of land available in Nigeria if well utilized, will make Nigeria to be a good source of artemisinin which is now a global remedy for the treatment of severe malaria.

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REFERENCES

1. Winstanley PA, Ward SA, Snow RW. Clinical status and implications of antimalarial drug resistance. *Microbes Infect*.2002; **4**: 157–164.
2. Klayman DL. Qinghaosu (artemisinin): An antimalarial drug from China. *Science*. 1985; **228**:1049–1055.
3. O'Neill PM. A worthy adversary for malaria. *Nature*, 2002; 430, 19. www.nature.com/nature
4. Nurgü;n E, Orhan k, Kartal M, Adigü;zel N, Bani B. Determination of Artemisinin in Selected *Artemisia* L. Species of Turkey by Reversed Phase HPLC. *Rec. Nat. Prod*. 2007; **1**:2–3, 36–43
5. WHO congress backs traditional medicine. *Nature*. 2008; **456**: 152.
6. Bilia AR, Melillo P, Malgalhaes D, Bergonzi MC, Vincieri FF. Simultaneous analysis of artemisinin and flavonoids of several extracts of *Artemisia annua* L. obtained from a commercial sample and a selected cultivar. 2006; *Phytomed*. **13**: 487–493.
7. Kohler M, Haerdi W, Christen P, Veuthey JL. Supercritical fluid extraction and chromatography of artemisinin and artemisinic acid. An improved method for the analysis of *Artemisia annua* samples. *Phytochem. Anal*. 2006; **8**: 223–227.
8. David C. Campaign to fight malaria hit by surge in demand for medicine. *Nature Publishing Group*. 2004; **432**: 259.
9. De Vries PJ, Dien TK. Clinical pharmacology and therapeutic potential of artemisinin and its derivates in the treatment of malaria. *Drugs*.1996; **52**:818–836.
10. Samuelsson G. *Drugs of Natural Origin*. 4th Revised edition, Swedish Pharmaceutical Press, Kristiandstad, Sweden. 1999
11. Klayman DL, Lin AJ, Acton N, Scovill JP, Hoch JM, Milhous WK, Theoharides AD, Dobek AS. Qinghaosu (artemisinin): An antimalarial drug from China. *J. Nat. Prod*. 1984; **47**: 715–717.
12. Peng CA, Ferreira JFS, Wood AJ. Direct analysis of artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector. *J. Chromatog, A* 2006; **1133**: 254–258.
13. Qian GP, Yang YW, Ren QL. Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC. *J. Liq. Chrom. & Rel. Technol*.2005; **28**: 705–712.
14. ElSohly HN, Croom EM, ElSohly MA. Analysis of the antimalarial sesquiterpene artemisinin in *Artemisia annua* by high-performance liquid chromatography (HPLC) with post column derivatization and ultraviolet detection. *Pharm. Res*. 1987; **4**: 258–260.
15. Woerdenbag HJ, Pras N, Bos R, Visser JF, Hendriks H, Malingre TM. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. *Phytochem. Anal*. 1991; **2**: 215–219.

Figure 3: *Representative chromatogram of nine determinations of Artemisia annua triplicate extract solution* (RT=3.931). Peak area: 24526.

Biochemical Composition of Seaweeds along Central West Coast of India

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ABSTRACT

Seaweeds from Asia have a great commercial application as the source of phycocolloides since a very long time. Present investigation reveals biochemical composition of three commonly occurring seaweeds, along the central west coast of India. Seaweeds viz. *Ulva fasciata* (green), *Sargassum ilicifolium* (brown) and *Gracillaria corticata* (red) were analyzed for protein, lipid, carbohydrate, ash and mineral content. *S. ilicifolium* ranked highest in total protein content among the species analyzed. All species contained approximately 5% crude lipid, although *U. fasciata* species had 6.3% crude lipid based on dry weight. Total carbohydrates ranged from 4.2 to 6.72% dry weight. Ash values ranged from 7.5% to 15.9%. All species contained measurable quantities of 10 essential mineral elements.

Keywords: *Protein, Seaweeds, Ulva fasciata*
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INTRODUCTION

Marine algae are looked upon as a renewable energy source, particularly from nutraceutical, biofuel and biofertilizer, point of views. India's coastline of ~7,500 km, greatly differs in its geomorphological and hydrological characters at various latitudes. The mid, intertidal and shallow subtidal regions, (particularly sandy and rocky), favour the growth of marine algae. Seaweeds though rich in vital chemicals of commercial importance, their utilization in India remain far below optimum level, compared to the same in other countries like Japan, South Korea, and China.

In the present study, biochemical properties of three seaweeds viz. *Ulva fasciata*, *Sargassum ilicifolium* and *Gracillaria corticata* was recorded by analyzing organic and inorganic content in them.

Seaweeds found to be good sources of proteins, carbohydrates, vitamins, and minerals in human nutrition. The marine algae contain various carbohydrates different from those in higher land plants, and in addition, high protein content has been reported [1] The central west coast is rich in algae, in terms of both biomass and algal species; however, there is no much information on the organic and inorganic compounds from this species. Hence, we analyzed other organic and Inorganic compounds in this species collected from the Kunkeshwar, Arabian Sea.

MATERIAL AND METHODS

Organic constituents:

Total carbohydrate content was estimated following anthron method [2] with slight modification. Amount of total carbohydrates was calculated with the help of a standard curve of sugar obtained by using different concentrations of standard glucose (0.1 mg/ml).

Total soluble proteins were estimated from the fresh thalli of seaweeds according to the method [3]. Amount of soluble proteins was calculated with the help of a standard curve obtained by using different concentrations of bovine serum albumin (0.1 mg/ml) by a similar procedure as employed for the plant extract. From air dried algal sample total lipid content was determined [4].

The mineral content was determined by charring a known amount of dry biomass in crucible in a furnace at 540°C for 5 hrs. The total mineral content (ash content) was then determined gravimetrically and expressed as percentage on dry weight basis.

Inorganic constituents:

For the determination of mineral elements, acid digest was prepared following the method [5]. For this oven, dried seaweed material was use. Macro and micronutrients were determined from this acid digest using an atomic

Table 1: Organic constituents in seaweeds

Sr. No.	Parameters	Ulva fasciata	Sargassum ilicifolium	Gracillaria corticata
1	Total Carbohydrates	5.92 ± 0.02	6.72 ± 0.02	4.20 ± 0.11
2	Total Proteins	3.45 ± 0.02	3.86 ± 0.02	3.30 ± 0.01
3	Total Lipids	6.3 ± 0.05	5.7 ± 0.03	4.2 ±0.02
4	Total Minerals	7.5 ± 0.2	15.9 ±0.05	10.8 ± 0.03

All values in percentage.

absorption spectrophotometer (Perkin-Elmer 3030 model). Trace elements Manganese, Copper, Zinc, and Nickel were also measure-using AAS from the same acid digest. Nitrogen was estimated using method[6]. Phosphorus content was estimate according to the method [7].

RESULTS AND DISCUSSION

Organic content in the three species of seaweeds is presented in Table 1. Total carbohydrate content varied from 4.20 to 6.72g 100^{-1}g dry weight, the maximum being recorded in *Sargassum* a brown alga and minimum was found in *Gracillaria,* a member of Rhodophyceae.

Carbohydrates comprise 50-60% of the dry weight of seaweeds[8]. Soluble carbohydrate reported 8.1-33.7% in *Enteromorpha*, *Ulva* and *Porphyra* [9]. The carbohydrate concentration of seaweeds varied from 20 – 24% [10]. The decrease in carbohydrates may be observed due to extensive growth of thallus of algae [11]. In general green seaweeds are reported to have a high carbohydrate level than those of red [12].

Total protein content in three species of marine algae did not vary much in different species and ranged from 3.3 to 3.8 g100^{-1}g dry wt. A protein content of less than 10% has been reported from the species of *Sargassum* and *Gracillaria* [13]. Significant variation in protein in the same species of algae growing at different localities and different periods [14].

Usually high protein content (10–47%) has been reported in red seaweeds [15]. while in green seaweeds it is fairly low [16].

Total lipid content of *Ulva, Sargassum* and *Gracillaria* from west coast of Maharashtra ranged from 4.2 to 6.3 %, highest being in brown alga *Sargassum* (Table 1). Lipid content in sea vegetables is very low, ranging from 1-5% of dry matter [17].

Ash analysis of the three seaweeds revealed a higher percentage of total minerals in Sargassum *ilicifolium* than in Ulva *fasciata* and *Gracillaria corticata*. A greater range in ash values and high ash content has been reported in the green algal species than in brown [10].

Total nitrogen content in three seaweed species varied from 9.08 to 14.35% being maximum in *Ulva fasciata;* however phosphorus content was high in *Gracillaria corticata* than the other two species. The values of potassium were also found to be at a higher side (Table 2).

Calcium content exhibited a little variation in different seaweeds but the Magnesium level was more or less similar.

Table 2: Inorganic constituents in seaweeds

Element*	Ulva fasciata	Sargassum ilicifolium	Gracillaria corticata
N	14350	9140	9080
P	3125	3500	5500
K	3125	4640	2819
Ca	3940	4320	4337
Mg	3478	3348	3568
Fe	05.38	18.34	13.76
Mn	02.30	09.62	03.64
Cu	01.16	01.20	00.92
Zn	04.52	06.40	0.96
Ni	oo.74	01.20	01.02

All values in mg/100g.dry wt

Iron content was significant in all the species. Manganese and zinc values varied in different species but copper and nickel concentration was minimum in all the seaweeds.

CONCLUSION

Present study revealed that they are affluent in essential macro, micronutrients and organic compounds required for nutritional purpose.

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REFERENCES:

1. Arasaki, S. and Arasaki, T. *Vegetables form the sea*. Japan Publication International, Tokyo, 196, (1983).
2. Sadasivam, S. and Manickam, A. *Biochemical method for agriculture science*, Willey, Eastern Ltd. **105** (1992).
3. Lowry, O. H., Rosebrough, N.J. and Randell, R.J. Protein measurement with the Folin phenol reagent. *J.Biol.Chem*, **193**: 265–275 (1951).
4. Bligh, E .G. and Dyer, W .J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. **37**: 911–917, (1959).
5. Toth, S.J., Prince, A. L, Wallace, A and Mikkelsen D.S. Rapid quantitative determination of eight mineral elements in plant tissue by systematic procedure involving use of flame photometer. *Soil Sci*., **66**: 459–466 (1948).
6. Hawk, P.B., Oser, B. L. and Summerson, W. H. *Practical physiological chemistry*. (Publ.) The Blackiston Co. USA. **1323** (1948).
7. Moller, M. and Smith, M. L. The effects of priming treatments using seaweed suspensions on the water sensitivity of barley (*Hordeum vulgare* L.). *Annal Appl. Biol*. **135**: 515 – 521(1999).
8. Sekine, T., Sasakawa, T., Morita, S., Kimura, T. and Kuratom, K. *cf. laboratory manual for physiological studies of Rice* (Eds.) Yoshida S., Forno D., Cook J.B. and Gomez K. A. (Pub.) International Rice Research institute, Manila, India. (1965).
9. Kennish, R. and Williams, G. A. Feeding preference of the herbivorous crab *Grapsus albolineatus:* the differential influence of algae nutrient content and morphology. *Mar. Ecol. Progr. Ser.*, **147**: 87–95 (1997).
10. Manivannan, K., Karthikai Devi, G., Thirumaran, G. and Anantharaman, P. Mineral composition of marine macroalgae from Mandapam coastal regions; southern coast of India. *Am.Eur.J. of Bot.*, **2**(1) 42–51 (2009).
11. Dhargalkar, V. K. *Biochemical studies on Ulva reticulata Forsskal*. Proc. Int. Symp. Marine algae of Indian Ocean region, CSMCRI, Bhavnagar, **40** (1979).
12. Pattama R and Chirapart, A. Nutritional evaluation of tropical green seaweeds *Caularpa lentillifera* and *Ulva reticulata*. *Kasetsart J. Nat. Sci.*, **40** (1): 75–83 2006.
13. Chidambaram, K. and Unny, M.M. Note on the value of seaweeds as manure. *I Intl. Seaweed Symposium*, 67–68 (1953).
14. Dave, M. J. and Parekh, R.G. Protein content of green seaweeds from the Southern coast. *Salt Res. and Ind.*, **11**(2): 41–44 (1975).
15. Wong, K.H. and Cheung, P.C.K. Nutritional evaluation of some subtropical red and green seaweed: Part I- Proximate composition, amino acid profiles and some physico-chemical properties. *Food chem.*, **71**: 475–482 (2000)
16. Dhargalkar, V. K. Biochemical studies on *Ulva reticulata* Forsskal, *Mahasagar Bull of the National Institute of Oceanography*, **19** (1): 45–57 (1986).
17. Haroon, A.M. The biochemical composition of *Enteromorpha* spp. From the Gulf of Gdansk coast on the Southern Baltic Sea. *Oceanologia*, **42**:19–28 (2000).

Screening of Some Coastal Plant Resources for Their Antioxidant Potential, Total Polyphenol and Flavonoid Content.

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Abstract

The methanolic extract of young and mature leaves of nine coastal plants from West coast of Maharshtra were analysed to evaluate free radical scavenging activity. The antioxidant activity was evaluated using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) and the reductive potential of the methanolic extract. The highest antioxidant activity was recorded in young leaves of *Hibiscus tiliaceus* (76 %), followed by *Syzigium corymbosa* (71 %) *Calophyllum inophyllum* (68 %) and *Colubrina asiatica* (55 %). The plant species also posseses appreciable reductive potential. The leaves of these species were found to be rich in Flavanoids (6.03 to 16.63 mg /g of dry weight) and total polyphenol (12.12 to 26.23 mg /g of dry weight) and these compounds mainly contributed the antioxidant potential of these plants.

Keywords: Antioxidants, Free radical, Reducing potential, phenolic content, coastal plants, Flavonoids.

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INTRODUCTION

Besides supporting human life by being a major source of food, plants also take care of human health. The antibacterial and antifungal properties of several plants are very well recognized (1-2). Plants are the potential source of natural antioxidants. Thus, antioxidants has an ability to protect the body against damages by free radical induced oxidative stress. The superoxide anions, hydrogen peroxide, hydroxyl ions, nitric oxide and peroxynitrite radicals are the Reactive Oxygen Species (ROS) and play an important role in oxidative stress related to pathogenesis of various important diseases (3-4). It is now very well realized that oxidative stress in an ultimate outcomes of various metabolic disorders in the cell and it is also a major casual factor for the degeneration and death of the cell.

Apart from their role of health benefactors, antioxidants are also added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors suchp as air, light and temperature (5). The antioxidant potential of the plant based compounds are often claimed for the protective effects of plant based beverages against cardiovascular diseases, certain form of cancer and photosensitivity reactions (6). Phenolics are antioxidant with redox potential which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (7). Phenolic compounds with antioxidant activity, are widely distributed in many fruits, vegetables, and tea are believed to account mainly for the antioxidant capacity of many plants (8-10). Over the past few years, investigations for phenolic compounds in medicinal herbs have gained importance due to their high antioxidative activity (11). Bandaranayake (12) stated that though mangroves and halophytes are extensively used in traditional medicine, only some of them were tested for biological activities and very few were studied their antioxidant properties.

The review of literature and the preliminary survey carried out by the authors in Ratnagiri and Sindhudurga districts of Maharashtra indicates that some coastal plant species are traditionally used for treatment of some human diseases, as well as in pest control. This is briefly summarized in Table. 1. In the present investigation an attempt has been made to study the antioxidant potential of the leaves of these species.

Table 1

Plant Name & Abbreviation	Location	Family	Ethnobotanical/ traditional uses	Parts used
Hibiscus tiliaceus (HB)	Ratnagiri	Malvaceae	Cool fever & sooth cough	Young and Mature Leaves
Calophyllum inophyllum (CP)	Malvan	Clusiaceae	skin inflammations, leg ulcers, wounds.	Young and Mature Leaves
Salvadora persica (SP)	Malvan	Salvadoraceae	Leaf fibers are used to treat eye-infection.	Young and Mature Leaves
Syzigium corymbosa (SC)	Aronda	Myrtaceae	---	Young and Mature Leaves
Colubrina asiatica (CA)	Aronda	Rhamnaceae	used medicinally and its leaves have long been used for soap	Young and Mature Leaves
Derris trifoliata (DT)	Aronda	Leguminosae	Leaves are used to stun or kill fish and shrimp	Young and Mature Leaves
Vitex negundo (V)	Ratnagiri	Verbenaceae	rheumatic swellings of the joints and in sprains. leaves are astringent, febrifuge, sedative, tonic and vermin fuge.	Young and Mature Leaves
Canavalia rosea (CN)	Aronda	Leguminosae-Papilionoideae	Chemical(s) found in plant shown to be effective for the ailment medicated	Young and Mature Leaves
Vitis trifoliata (VT)	Ratnagiri	Vitaceae		Young and Mature Leaves

MATERIAL AND METHODS

Plant materials of ten coastal taxons were collected from the west coast of Maharashtra (India). Particulars of these sources including species, families, and their uses have been listed in Table 1.

Dried Leaves (1 g each) was macerated with 100 ml methanol for 48 h at room temperature. Filtration and collection of the extract was done thrice. Then, the methanol with crude extract (300 ml) was evaporated to 10 ml and dried on water bath at 40°C. The dry extract was weighted and dissolved in methanol (stock 5mg/ml) and stored in -20°C until further use.

Free radical scavenging activity determination

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (13). Fifteen hundred µl of each plant extract were added, at an equal volume, to methanolic solution of DPPH(100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHT and Ascorbic acid were used as standard controls. The percentage of DPPH discoloration of the sample was calculated according to the equation: % discoloration = (1-Abs sample /Abs control) × 100. The Ascorbic acid and BHT were used for the positive control in the aqueous extract and ethanolic extracts respectively.

Determination of Reducing power

The reducing power of the extracted samples (dissolved in ethanol or distilled water), Ascorbic acid (dissolved in distilled water) or butylated hydroxytoluene (BHT, dissolved in ethanol) were determined according to the method of Jayaprakasha et al. (14). Five hundred µl of each extract, AA or BHT were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, to reduce ferricyanide into ferrocyanide. Thereafter, an equal volume of 1% trichloroacetic acid was added to the mixture and centrifuged at 6,000 rpm for 10 min. The upper layer of the solution was collected and mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:0.2. Absorbance was measured to determine

the amount of ferric ferrocyanide (Prussian Blue) formed at 700 nm against a blank in a UV/Vis double spectrophotometer (Sysctronics). The AA and BHT were used as positive control for aqueous extract and ethanolic extracts respectivelly. The reducing power tests were run in triplicate. Increase in absorbance of the reaction indicated the reducing power of the samples.

Total Polyphenol Content (TPC)

Total phenolic compounds were estimated using the Folin-Ciocalteu method of Ragazzi and Veronese (15). Twenty µl of each extract was added to 500 µl distilled water and 125 µl of Folin-Ciocalteu phenol reagent. The mixture was allowed to stand at room temperature for 10 min, and then 1.25 ml of 20% sodium carbonate was added to the mixture. The final volume was made 3 ml by adding distilled water. The mixture was incubated for 90 min and the resulting blue complex was then measured at 760 nm. The TPC was expressed as mg gallic acid equivalent/g dry weight by reference to the (+)- gallic acid standard calibration curve.

Total flavonoid content

The $AlCl_3$ method of Lamaison and Carnet (16) was used for estimation of the total flavonoid content of the extracted samples. An aliquot of 1500 µl of each extract was added individually to equal volumes of 2% $AlCl_3$ $6H_2O$ (2 g in 100 ml methanol). The mixture was vigorously shaken, and after 10 min of incubation, absorbance was taken at 367 nm. Flavonoids contents were calculated from the calibration curve of Quercetin standard solutions, and expressed as mg Quercetin equivalent/ g dry weight.

Statistical analysis

All data were reported as mean ± standard deviation of three replicates. The results were compared by one-way analysis of variance (ANOVA) and Tukey's test were carried out to test any significant differences among the means using Graph pad instat software. Differences among means ($P < 0.001$) were considered statistically significant.

RESULTS AND DISCUSSION

The free radical scavenging activity was represented on the basis of percent inhibition. It is evident from the fig. (1) that the young leaves of all the investigated species except *Derris trifolaita* possesses higher antioxidant activity than the mature leaves. The highest free radical scavenging activity recorded in *H. tiliaceus* i.e. 76 %, The leaves of *S. corymbosa* (71 %), *C. inophyllum* (68 %) also shows appreciable free radical scavenging activities. The antioxidant activity in the young and mature of *Canavalia rosea* and mature leaves of *C. asiatica* are rather low.

Leaf extracts of ten coastal plants sources exhibited the reducing power (Fig. 2). Higher absorbance represents higher reductive potential of the sample. An interspecific variation in the reducing potential is clearly noticed in the present investigation. It was found that the young leaves *H. tiliaceus* (1.26) had a higher degree of reducing power followed by *C. inophyllum* (1.14) and *S.*

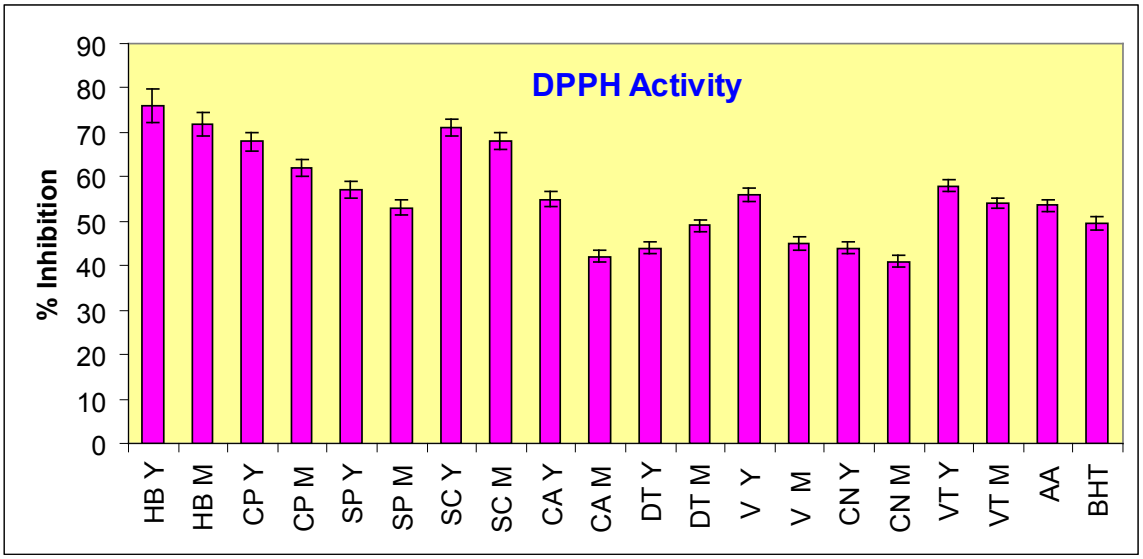

Figure 1. Antioxidant activity (% ± S.D.) of methanolic extract of young and mature leaves 10 different taxons of coastal bioresources measured using DPPH.

corymbosa (1.04). The mature leaves of *Vitis* (0.207). *D. trifoliata* (0.268) and *S. persica* (0.253) had lower reductive potential. According to Duh (17), the reducing properties are generally associated with the presence of reductones. Gordon (18) have reported that the antioxidant action of reductones was based on the cleavage of free radical chain by hydrogen atom donation.

The levels of total polyphenol and total flavonoid content in the leaves of different coastal plants are given in Fig-3 and 4. The total polyphenol contents of these plants ranges from 12.12 to 26.23 mg/g dry tissue. The highest polyphenol content (26.23 mg/g) was recorded in young leaves of *H. tiliaceus*. Comparable values are also recorded in case of young leaves of *S. corymbosa* (24.68 mg/g) and *C. inophyllum* (21.67 mg/g). The difference in polyphenol level in the two stages of leaf development dose not show a uniform trend in case of the species studied. Thus, the young leaves of *Hibiscus tiliaceus, Calophyllum inophyllum* and *Syzigium corymbosa* show higher level of polyphenols than the mature leaves while opposite trend is noticeable in case of *Salvadora persica, Colubrina asiatica* and *Canavalia rosea*.

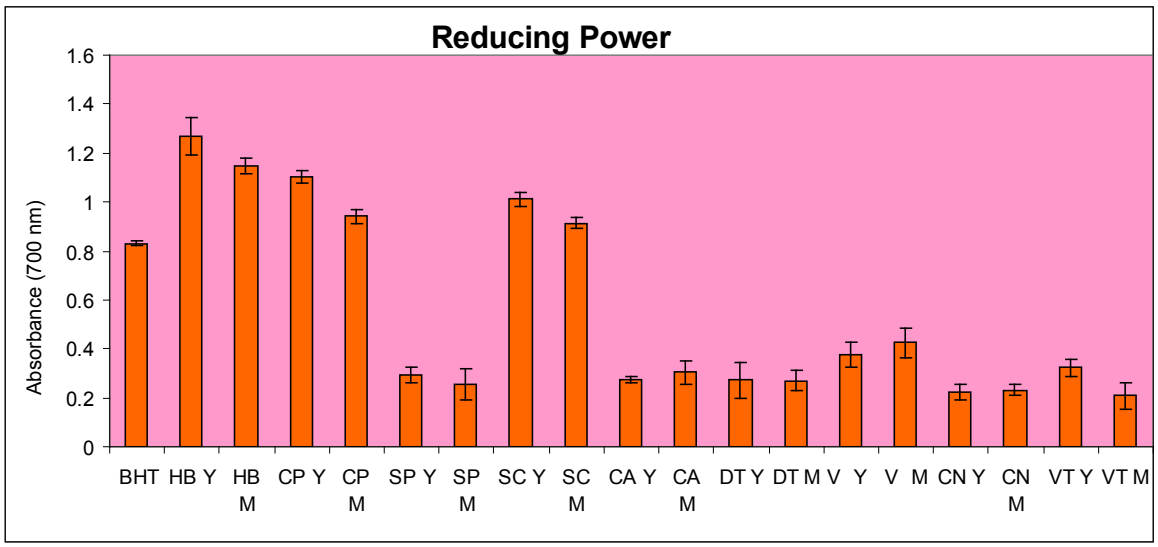

Figure 2. Reducing power of methanolic extract of young and mature leaves of 10 different taxons of coastal bioresources and BHT (± S.D).

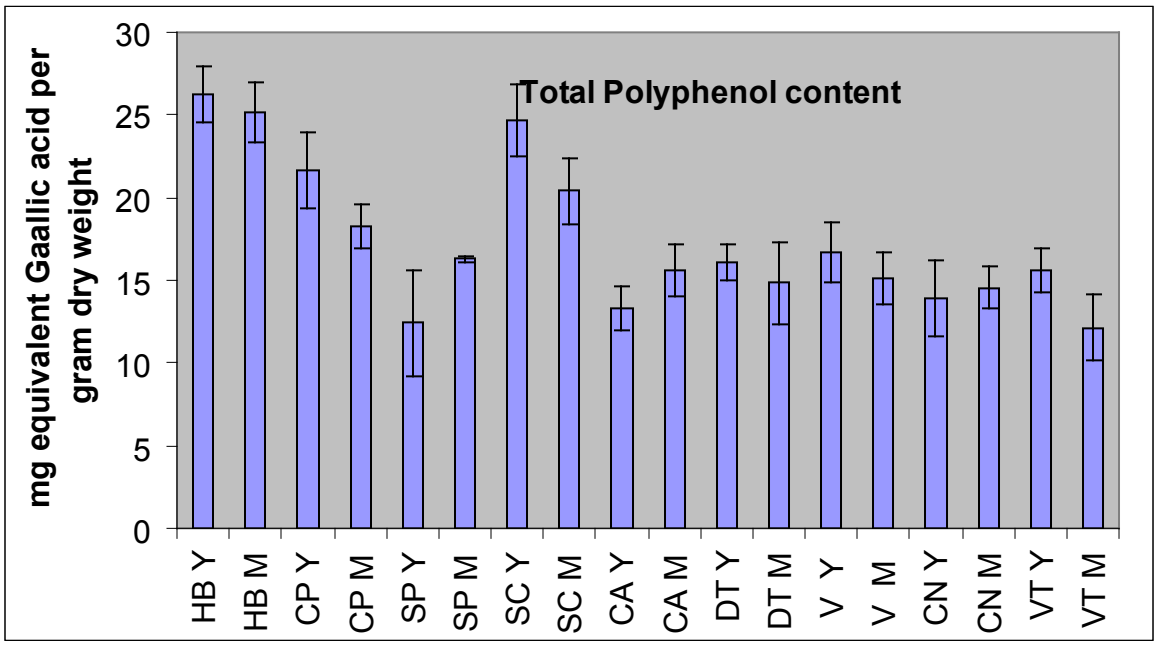

Figure 3. Total polyphenol content of the young and mature leaves of the 10 coastal bioresources (± S.D)

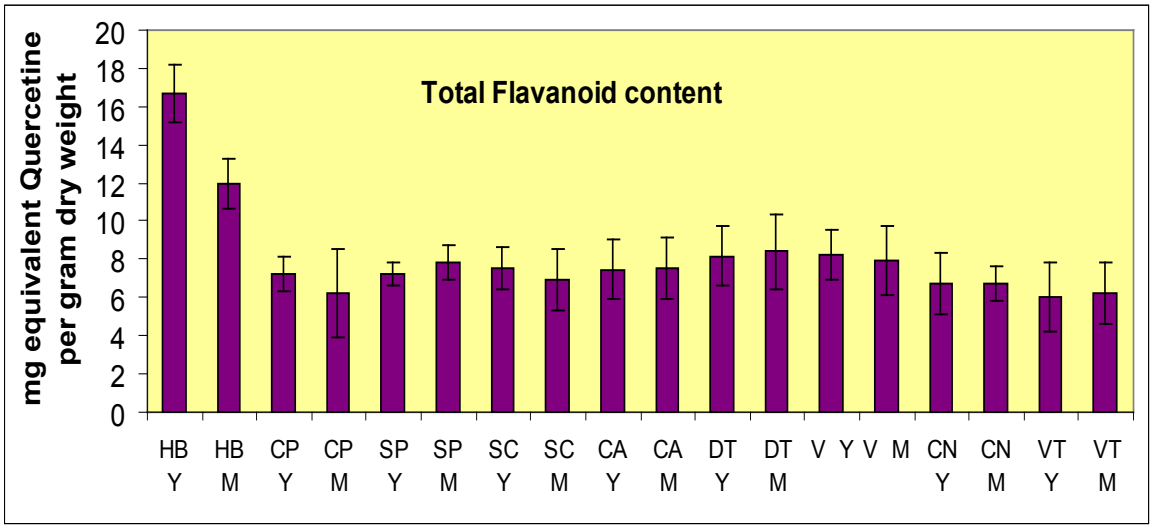

Figure 4. Total flavonoid content of the young and mature leaves of the 10 coastal bioresources (± S.D)

Flavonoids form an important group of phenolic compounds. The highest total flavonoid content was recorded in young leaves of *H. tiliaceus* (16.659 mg/g dry tissue) while mature leaves of *C. inophyllum* show lo west level of flavonoids. In six species the flavonoid level in the young leaves is higher than in mature leaves while in *S. persica* (7.866 mg/g dry tissue) opposite trend is seen.

Mahabusarakam et al (19) and Minami et al (20) reported that the antioxidative properties of the extracts obtained from *Garcinia subelliptica* and *Garcinia manostana* were due to presence of various xanthones with phenolic functional groups. The relationship between phenolic constituents and antioxidants potential has been documented in several studies. Dimitrova et al (21) demonstrated the presence of Caffeic acid, rosaminic acid in *Melissa officinalis*. Kahkonen et al (22) characterized phenolic acids and flavonoids as typical phenolics having antioxidant capacity. Caffeic acid, ferulic acid and vanillic acid are considered as natural antioxidants as phenolic acid present in fruits, vegetables and other plants (23). According to Javanmardi et al (24) the antioxidant activity of phenolic is mainly due to their redox potentials which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Lu and Foo (25); Zheng and Wang (26) and Rice- Evans et al (27) have stated that the antioxidant activity of compounds from botanical origin is proportional to the phenolic content. According to Shahidi et al (28) phenolic antioxidants are potent free radical terminators. Sawa et al (29) has been reported that the potential of phenolics to scavenge free radicals may be due to phenolic hydroxyl groups. According to Svobodova et al (30) polyphenols are believed to provide a chemical defense against the predators and ultraviolet radiations. Oki et al (31) have also noticed that an increase in antioxidant activity was found to be associated with increased polyphenol content. Present study also reveals similar trend especially with respect to young and mature leaves of *Hibiscus tiliaceus, Syzigium corymbosa, Vitex negundo*, and *Calophyllum inophyllum*. Our findings further support the validity of inclusion of these species in the traditional medical practices for treatment of various diseases.

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REFERENCES

1. Okwu D.E. Evaluation of Chemical Composition of indigenous species and flavouring agents. *J Pure Appl Sci* **7**: 455–459 (2001).
2. Urugulaga I. and Laghton F. Plant polyphenol antioxidants and oxidative stress. *Biol Res* **33**: 159–165 (2000).
3. Halliwell B. and Gutteridge J.M.C. *"Free Radicals in Biology and Medicine"*. 3rd edition, (Publ.) Oxford University Press, Oxford, U.K. (1999).
4. Finkel T. and Holbrook N.J. Oxidants, oxidative stress and the biology of aging. *Nature* **408**: 239–247 (2000).
5. Hras A.R., Hadolin M., Knez Z. and Bauman D.. Comparison of antioxidative and synergistic effects of rosemary extract with alpha-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry* **71**:229–233 (2000).
6. Bravo L. Polyphenols Chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev* **56**: 317–333 (1998).
7. Pietta P.G. Flavonoids as antioxidants. *J Nat Prod* **63**:1035–1042 (2000).

8. Kaur C. and Kapoor H.C. Antioxidant activity and total phenolic content of some Asian vegetables. *Int J Food Sci Tech* **37**(2): 153–162 (2002).
9. Vinson J.A., Hao Y., Su X. and Zubik L. Phenol antioxidant quantity and quality in foods: vegetables. *J Agric Food Chem* **46**: 3630–3634 (1998).
10. Wu X., Beecher G.R., Holden J.M., Haytowitz D.B., Gebhardt S.E. and Prior R.L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J Agric Food Chem* **52**, (12) : 4026-4037 (2004).
11. Zhu Y.Z., Huang S.H., Tan B.K., Sun J., Whiteman M. and Zhu Y.C. Antioxidants in Chinese herbal medicines: a biochemical perspective. *Nat Prod Rep***21**: 478–489 (2004).
12. Bandaranayake W.M. Traditional and medicinal uses of mangroves. *Mangroves and Salt Marshes* **2**: 133–148 1998.
13. Koleva I.I., Van Beek T.A., Linssen J.P.H., de Groot A. and Evstatieva L.N. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis* **13**: 8–17 (2002).
14. Jayaprakasha G.K., Singh R.P. and Sakariah K.K.. Antioxidant activity of grape seed (Vitis vinifera) extracts on peroxidation models in vitro. *Food Chem* **73**: 285–290 (2002).
15. Ragazzi E. and Veronese G. Quantitative analysis of phenolics compounds after thin layer chromatographic separation. *J Chromotogr* **77**: 369–375 (1973).
16. Lamaison J.L.C. and Carnet A. Teneurs en principaux flavonoids desfleurs de Crataegeus monogyna Jacq et de Crataegeus laevigata (Poiret D. C) en function de la vegetation. *Pharm Acta Helv* **65**: 315–320 (1990).
17. Dhu P.D. Antioxidant activity of Budrock (Arctium lappa Linn.) its scavenging effect on free radical and active oxygen. *J Am Oil Chem Soc* **75**: 455–461 (1998).
18. Gordon M.F. The mechanism of antioxidant action in vitro. In Hudson B.J.F.(ed.), Food Antioxidants. (Publ.) Elsevier Applied Science, London, pp.1–18. (1990).
19. Mahabusarakam W., Proudfoot J., Taylor W. and Croft K. Inhibition of lipoprotein oxidation by prenylated xanthones derived from mangostin. *Free Radic Res* **33**: 643–659 (2000).
20. Minami H., Kuwayama A., Yoshizawa T. and Fukuyama Y. Novel prenylated xanthones with antioxidant property from the wood of Garcinia subelliptica. *Chem Pharm Bull* **44**: 2103–2106 (1996).
21. Dimitrova Z., Dimov B., Monolova N., Pancheva S., Ilieva D. and Shishkov S. Antiherpes effect of Melissa officinalis L. extracts. *Acta Microbio Bulg* **29**:65–72 (1993).
22. Kahkonen M.P., Hopia A.I., Vuorela H.J., Rauha J.P., Pihlaja K., Kujala T.S. and Heinonen M. Antioxidant activity of plant extracts containing phenolic compounds. *J Agric Food Chem* **47**: 3954–3962 (1999).
23. Larson R.A. The antioxidants of higher plants. *Phytochemistry* **27**: 969–978 (1988).
24. Javanmardi J., Khalighi A., Kashi A., Bais H.P. and Vivanio J.M. Chemical characterization of basil (*Ocimum basiliam* L.) found in local accessions and used in traditional medicines in Iran. *Journal of Agriculture and Food Chemistry* **50**: 5878–5883. (2003).
25. Lu Y. and Foo Y. L. Antioxidant and free radical scavenging activities of selected medicinal herbs. *J Life Sci* **66**: 725–735 (2000).
26. Zheng W. and Wang S. Antioxidant activity and phenolic composition in selected herbs. *J Agric Food Chem* **49**(11):5165–70 (2001).
27. Rice-Evans C.A., Miller N.G. and Paganga G. Antioxidant properties of phenolic compounds. *Trend Plant Sci* **2**: 152–159 (1997).
28. Shahidi F., Janitha P.K. and Wanasundara P.D. Phenolic antioxidants. *Crit Rev Food Sci Nutr* **32**: 67–103(1992).
29. Sawa T., Nakao M., Akaike T., Ono K. and Maeda H. Alkylperoxyl radical scavenging activity of various flavonoids and other phenolic compounds: Implications for the antitumor promoter effect of vegetables. *J Agric Food Chem* **47**: 397–492 (1999).
30. Svobodova A., Psotova J. and Walteora D. Natural phenolics in the prevention of uv-induced skin damage. A Review. *Biomed Papers* **147**(2): 137–146. (2003).
31. Oki T., Masuda M., Furtura S., Nishibia Y., Terahara N. and Suda I. *J Food Chem Toxicol* **67**: **1752**. (2002).

In Vitro Antioxidant Activity Of Methanolic Extract Of *Rhodiola Imbricata* Edgew.

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Abstract

Rhodiola imbricata (Roseroot) is a perennial plant distributed in South Asia viz., Pakistan, Nepal, China and India. In India it is endemic to Trans Himalayan cold desert of Ladakh. It is a well known medicinal plant in the Amchi system of medicine (Tibetan system of medicine) being used in various ailments like anti-stress, radio-protective, anticancer, anti-inflammatory agent, adaptogen etc. The aim of this study was to assess the in vitro potential of methanol extract of *Rhodiola imbricata* roots. The DPPH activity of the extract (0.1–1.2 mg/ml) was increased in a dose dependent manner, which was found in the range of (39.55-70.76%) as compared to ascorbic acid (46.78 –81.47%). The IC_{50} values of methanol extract in DPPH radical, nitric oxide, hydroxyl radical were obtained to be 0.33, 0.47, 0.58 mg/ml, respectively. However, the IC_{50} values for the standard ascorbic acid were noted to be 0.42, 0.43, 0.51 mg/ml, respectively. Measurement of total phenolic content of the methanol extract of *R. imbricata* was achieved using Folin–Ciocalteau reagent containing 185.7 mg/g of phenolic content, which was found significantly higher when compared to reference standard gallic acid. The results obtained in this study clearly indicate that *R. imbricata* has a significant potential to use as a natural anti-oxidant agent.

Keywords: Antioxidant; DPPH; Hydroxyl radical; Nitric oxide radical; *Rhodiola imbricata*.

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INTRODUCTION

Research on relationships between antioxidants and prevention of non-communicable disease, such as cardiovascular disease, cancer and diabetes has been increasing sharply in recent years. Free radicals have been claimed to play a key role, affecting human health by causing severe diseases, such as cancer and cardiovascular diseases by cell degeneration. These free radicals can be generated during normal body function, and can be acquired from the environment. Interestingly the body possesses defence mechanism against free radical-induced oxidative stress, which involves preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses. Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Majority of the diseases/disorders are mainly linked to oxidative stress (1). The most common reactive oxygen species (ROS) include superoxide (0_2), hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and reactive hydroxyl (OH) radicals. The nitrogen derived free radicals are nitric oxide (NO) and peroxynitrite anion (ONOO). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (2). Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) etc. Whereas non-enzymatic antioxidants are ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids, flavonoids etc (3). All these act by one or more mechanisms like reducing activity, free radical-scavenging, potential complexing of pro-oxidant metals and quenching of singlet oxygen. It is possible to reduce the risks of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with proven dietary antioxidants. This is one of the reasons why discovery and synthesis of novel antioxidants is a major active area. Synthetic antioxidants

like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) commonly used in processed foods have side effects and are carcinogenic (4). In recent years, the use of natural antioxidants present in food and other biological materials has attracted considerable interest due to their presumed safety, nutritional and therapeutic value. Epidemiological and *in vitro* studies strongly suggest that food containing phytochemicals with antioxidants have potentially protective effects against many diseases, including cancer, diabetes and cardiovascular diseases (5).

Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability (6). Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic. etc. (7), they were also suggested to be a potential iron chelator (8, 9).

Rhodiola imbricata (Roseroot, Artic root, or Shrolo), belonging to the stone crop family *Crassulaceae*, is an important food crop and medicinal plant in Trans-Himalayan cold desert (10, 11). It is a popular medicinal plant in India, Tibet, China Nepal and Pakistan (12). *Rhodiola imbricata* is mainly known for its number of physiological functions including neurotransmitter levels, central nervous system stimulating activity, cardiovascular function, depression decreasing, enhancing work performance and learning, eliminate fatigue, immunomodulatory properties, and to prevent high-altitude sickness (13–15). Considering the importance of this area, present study was focused on some important *in-vitro* evaluation of antioxidant activity and quantification of total phenolic compounds responsible for free scavenging activity.

MATERIAL AND METHODS

Collection and Identification of Plant material

Roots of *R. imbricata*, were collected from Trans-Himalayan region (Chang-La Top, altitude 17500 ft. Above Mean Sea Level), in India in the month of June. The collected roots were identified and authenticated by a scientist Dr. OP Chaurasia, Medicinal and Aromatic Plant Division, Defence Institute of High Altitude Research (DRDO), C/o 56 APO, India. A Voucher specimen (Specimen no: A - 3) has been deposited at the Herbarium of our division. The plant samples were washed thoroughly to remove clay and dirt from them. The roots were cut into small pieces and shade dried at room temperature for 15 days, finely powdered and used for extraction.

Preparation of root extract

R. imbricata root powder was successively extracted by methanol with the help of Soxhlet apparatus till the residue remains colourless. The obtained extract was concentrated using rotary evaporator under vacuum and reduced pressure at 40°C and the residue was used for further studies.

Quantification of total polyphenolic compounds

Total polyphenols were determined by the Folin–Ciocalteu procedure (16). Aliquots (0.1 ml) of test-solution were transferred into the test tubes and volumes brought up to 0.5 ml by water. After addition of 0.25 ml Folin–Ciocalteu reagent and 1.25 ml 20% aqueous Na_2CO_3 solution, tubes were vortexed and absorbance of blue-coloured mixtures recorded after 40 min at 725nm against blank, containing 0.1 ml of extraction solvent. The amount of total polyphenols was calculated from the calibration curve of gallic acid standard solutions, concentration of total phenols was expressed as mg/g of dry extract.

DPPH radical scavenging activity

The free radical scavenging activity of the sample was measured *in vitro* by DPPH (1, 1 diphenyl 2, picryl hydrazyl) assay (17). The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH. 0.1mM solution of DPPH in methanol was prepared and 3.0 ml of this solution was added to 40.0 µl of extract solution in water at different concentrations (200–1200µg/mL). The mixture was incubated at room temperature for 30 minutes and the absorbance was measured at 515 nm against corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated based on the control reading using the following equation:

$$\text{DPPH Scavenged (\%)} = (A_{cont} - A_{sample}) / A_{cont} \times 100$$

Where A_{cont} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the extract/standard.

The antioxidant activity of the extract was expressed as IC_{50}, which the concentration (in µg/ml) of extract inhibits formation of DPPH radicals by 50%.

Nitric oxide radical-scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside

in aqueous solution at physiological pH spontaneously generates nitric oxide (18) which interacts with oxygen to produce nitric ions that can be estimated by using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate buffer saline (PBS) was mixed with 3.0 ml of different concentrations (0.2-1.2 mg/ml) of the *R. imbricata* extract and incubated at 25^{0}C for 150 min. The samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 546 nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ inhibition} = (A_{cont} - A_{sample}) / A_{cont} \times 100$$

Where A_{cont} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the extract/ standard. All the tests were performed in triplicate and the graph was plotted with the mean values. Ascorbic acid was used as a positive control.

Hydroxyl radical-scavenging activity

The hydroxyl radical scavenging capacity was measured using modified method as described previously (19). Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled de-ionized water. The assay was performed by adding 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of extract (0.2–1.2 mg/ml) each dissolved in methanol, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37^{0}C for 1 h. 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of (10%) trichloroacetic acid and 1.0 ml of (0.5%) thiobarbituric acid (in 0.025 M NaOH containing 0.025 MNaOH butyl hydroxyl anisol) to develop the pink chromogen, which was measured at 532 nm. The hydroxyl radical-scavenging activity of the extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the following equation:

$$\% \text{ inhibition} = (A_{cont} - A_{sample}) / A_{cont} \times 100$$

Where A_{cont} is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the extract/ standard. All the tests were performed in triplicate and the graph was plotted with the mean values. Ascorbic acid was used as a positive control.

RESULTS AND DISCUSSION

Total Phenolic compounds

Phenolics are ubiquitous secondary metabolites in plants and possess a wide range of therapeutic uses such as antioxidant, antimutagenic, anticarcinogenic, free radical scavenging activities and also decrease cardiovascular complications (20). The content of phenolic compounds (mg/g) in methanolic extract was found 185 mg/g plant extract and expressed in gallic acid equivalents. These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The scavenging ability of the phenolics is mainly due to the presence of hydroxyl groups. The phenolic compounds may contribute directly to anti-oxidative action. It is known that poly phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans when ingested up to 1 g daily from a diet rich in fruits and vegetables (21). Phenolic compounds from plants are known to be good natural anti-oxidants. However, the activity of synthetic anti oxidants was often observed to be higher than that of natural anti-oxidants (22).

DPPH radical scavenging activity

The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 515 nm induced by antioxidants. On the DPPH radical, R. imbricata had significant scavenging effects with increasing concentration in the range of 0.2–1.2 mg/ml when compared with that of ascorbic acid; the scavenging effect of R. imbricata was lower. The IC_{50} values were found to be 0.33 and 0.42 mg/ml for R. imbricata and ascorbic acid, respectively (Table 1). The DPPH activity of R. imbricata was found to increase in dose dependent manner. The R. imbricata at the used concentrations displayed potential effect of DPPH activity as percentage of free radicals inhibition. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. These data clearly indicate that RHME is a powerful free radical inhibitor or scavenger.

Nitric oxide radical scavenging

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. In the present study the crude methanol extract of the R. imbricata was checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by R. imbricata. The R. imbricata at varied concentrations showed remarkable inhibitory effect of nitric oxide radical-scavenging activity (Table 2). Results showed the percentage of inhibition in a dose dependent manner. The concentration of R. imbricata needed for 50% inhibition (IC_{50}) was found to be 0.47 mg/ ml, whereas 0.43 mg/ml was needed for ascorbic acid. The results were found to be statistically significant.

Hydroxyl radical scavenging

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (23). The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The effect of R. imbricata on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the Fe^{+2}-dependent DNA damage assay. The Fentone reaction generates hydroxyl radicals (OH) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component. Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. R. imbricata was also capable of reducing DNA damage at all concentrations used. Ascorbic acid was highly effective in inhibiting the oxidative DNA damage. As shown in Table 3, the R. imbricata displayed potential inhibitory effect of hydroxyl radical-scavenging activity. All results showed anti-oxidant activity in dose dependent manner. IC_{50} values were found to be 0.58 and 0.51 mg/ml for R. imbricata and ascorbic acid, respectively (Table 3).

CONCLUSION

It is well known that free radicals are one of the causes of several diseases, such as Parkinson disease, Alzheimer

"Table 1: DPPH radical-scavenging activity"

Conc. (µg/ml)	RHME	Linear equation (r^2)	Ascorbic acid	Linear equation (r^2)
200	46.78 ± 0.93		39.55±1.80	
400	54.89± 1.11		49.97±1.63	
600	62.24± 0.70	y = 4.6083x +45.107	60.05±0.88	y = 7.7333x +33.716
800	64.92± 0.73	r^2 = 0.9348	64.92±0.73	r^2 = 0.9726
1000	67.80± 2.02		68.70±1.92	
1200	70.76± 1.91		81.47±0.53	
	IC_{50}=.33mg/ml		IC_{50}=.42mg/ml	

RHME- Methanol extract of *Rhodiola imbricata*
Values are mean ± S.D (n=3)
P<0.05 Compared to control

"Table 2: Nitric oxide radical -scavenging activity"

Conc. (µg/ml)	RHME	Linear equation (r^2)	Ascorbic acid	Linear equation (r^2)
200	33.47±0.27		40.19±1.36	
400	46.80±0.33		47.79±0.16	
600	53.88±0.21	y = 8.8927x +31.805	58.81±0.21	y = 9.165x +26.423
800	62.50±0.27	r^2 = 0.9843	68.12±0.28	r^2 = 0.9798
1000	75.83±0.16		79.00±0.33	
1200	78.49±0.16		81.85±0.16	
	IC_{50}=.47mg/ml		IC_{50}=.43mg/ml	

RHME- Methanol extract of *Rhodiola imbricata*
Values are mean ± S.D (n=3)
P<0.05 Compared to control

"Table 3: Hydroxyl radical -scavenging activity"

Conc. (µg/ml)	RHME	Linear equation (r^2)	Ascorbic acid	Linear equation (r^2)
200	23.61±0.67		29.97±0.27	
400	32.18±0.16		37.39±0.16	
600	52.77±0.39	y = 10.901x +3.5955	60.79±0.41	y = 10.271x +11.847
800	62.23±0.16	r^2 = 0.9604	68.18±0.38	r^2 = 0.9281
1000	69.36±0.16		73.09±0.52	
1200	75.72±0.22		78.97±0.33	
	IC_{50}=.58mg/ml			IC_{50}=.51mg/ml

RHME- Methanol extract of *Rhodiola imbricata*
Values are mean ± S.D (n=3)
P<0.05 Compared to control

type dementia etc. The production of free radicals and the activity of the scavenger enzymes against those radicals such as super oxide dismutase (SOD) are correlated with the life expectancies. We have demonstrated the chloroform extract of R. imbricata roots contained high level of total phenolic compounds and were capable of inhibiting, quenching free radicals to terminate the radical chain reaction, and acting as reducing agents. Accordingly in this study, a significant and linear relationship was found between the anti-oxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to anti-oxidant activity. The methanol extract of R. imbricata roots showed strong anti-oxidant activity by inhibiting DPPH, nitric oxide, hydroxyl radical scavenging activities when compared with standard ascorbic acid. These results are very curious for further drug research and can help in developing new drugs related to stress induced ailments.

REFERENCES:

1. Gutteridgde J.M.C.. Free radicals in disease processes: A complication of cause and consequence. *Free Radic. Res. Comm.* **19**: 141–158 (1995).
2. Joyce D.A.. Oxygen radicals in disease. *Adv. Drug Reac. Bull.* **127**: 476–79 (1987).
3. Stanner S.A., Hughes J., Kelly C.N.. Review of the epidemiological evidence for the antioxidant hypotheses. *Pub Health Nutr.* **7**: 407 – 422 (2004).
4. Ali S., Kasoju N., Luthra A., Singh A., Sharanabasava H., Sahu A. and Bora U.. Indian medicinal herbs as sources of antioxidants. *Food Res Int.* **41**: 1 – 15 (2008).
5. Senevirathne M., Kim S., Siriwardhana N., Ha J., Lee K. and Jeon Y.. Antioxidant potential of Ecklonia cava on reactive oxygen species scavenging metal chelating, reducing power and lipid peroxidation inhibition. *Food SciTech Int.* **12** (1): 27 – 38 (2006).
6. Auudy B., Ferreira F., Blasina L., Lafon F., Arredondo F., Dajas R. and Tripathi P.C.. Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases. *J. Ethnopharmacol.* **84**: 131–138 (2003).
7. Miller A.L.. Antioxidant flavonoids: structure, function and clinical usage. *Alt. Med. Rev.* **1**: 103–111 (1996).
8. Boyer R.F., Clark H.M. and Laroche A.P.. Reduction and release of ferritin iron by plant phenolics. *J. Inorg. Biochem.* **32**: 171–181 (1988).
9. Havsteen B.. Flavonoids, a class of natural products of high pharmacological potency. *Biocchem. Pharmacol.* **30**: 1141–1148 (1983).
10. Chaurasia O.P., Singh B.. *Cold desert plants*. Vol. **I-IV**. Field Research Laboratory DRDO Leh. (1996).
11. Kumar G. P., Gupta S., Murugan P.M.. Ethnobotanical studies of Nubra Valley – a cold arid zone of Himalaya. *Ethnobot Leaf* **13**: 752–765 (2009).
12. Chaurasia O.P., Ahmed Z. and Ballabh B.. *Ethnobotany & Plants of trans-Himalaya*. Satish Serial Publishing House Delhi. (2007).
13. Kumar G.P. Floral wealth of Nubra valley. *Phytological Research*.**2**: (2009).(In press)
14. Mishra K.P., Padwad Y.S., Jain M., Karan D., Ganju L. and Sawhney R.C.. Aqueous Extract of Rhodiola imbricata Rhizome Stimulates Proinflammatory Mediators via Phosphorylated I B and Transcription Factor Nuclear Factor- B. *Immunopharmacology and Immunotoxicology*. **28** (2): 201–212(2006).
15. Kanupriya, D. Prasad, M. Sai Ram, R. Kumar, R.C. Sawhney, S.K. Sharma, G. Ilavazhagan, D. Kumar and Banerjee P.K..Cytoprotective and antioxidant activity of *Rhodiola imbricata* against *tert*-butyl hydroperoxide induced oxidative injury in U-937 human macrophages. *Molecular and Cellular Biochemistry*. **275**: 1–6 (2005)
16. Slinkard K., Singleton V.L.. Total phenol analyses: automation and comparison with manual methods. *American Journal of Enology and Viticulture* **28**: 49–55(1977)
17. Shimada K., Fujikawa K. and Nakamura T. Antioxidative properties of xanthone on the autooxidation of soyabean in cyclodextrin emulsion. *J. Agric. Food Chem.* **40**: 945–948(1992).
18. Marcocci L., Maguire J.J. and Droy M.T.. The nitric oxide scavenging properties of Gingo biloba extract EGb 761. *Biochemical and Biophysical Research Communications* **15**: 748–755 (1994).
19. Halliwell B., Gutteridge J.M.C.. *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford. (1999).
20. Yen G., Duh P. and Tsai C.. Relationship between antioxidant activity and maturity of peanut hulls. *J Agric Food Chem.* **41**: 67 – 70 (1993).
21. Tanaka M., Kuei C.W. and Nagashima Y.. Application of antioxidative Maillard reaction products from histidine and glucose to sardine products. *Nippon Suisan Gakkaishi*. **47**:1409–1414(1998).
22. Ningappa M.B., Dinesha R. and Srinivas L.. Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf extract (*Murraya koenigii L.*). *Food Chemistry*. **106**:720–728(2008).
23. Hochestein P. and Atallah A.S. The nature of oxidant and antioxidant systems in the inhibition of mutation and cancer. *Mutation Research*. **202**: 363–375(1988).

Determination of Polyphenolic Content and In-vitro Antioxidant Capacity of the Leaves of *Lagenaria siceraria* (mol.) standl

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Abstract

The leaves of *Lagenaria siceraria* (bottle gourd), belonging to the family cucurbitaceae being used for the treatment of jaundice and also claimed to be effective in many other diseases. The present research work was under taken to investigate the in-vitro antioxidantactivity of aqueous and ethanolic extracts. The free radicals are capable of independent existence and cause oxidative tissue damage. The therapeutic effects of tannins and flavonoids can be largely attributed to their antioxidant properties. So that the quantitative determinations were undertaken, the total Phenolic content of aqueous and ethanolic extracts showed the content values of 3.75 ± 0.22% w/w and 22.12±1.23%w/w respectively and total flavonoids estimation of aqueous and ethanolic extract showed the content values of 1.33±0.08%w/w and 3.61± 0.32%w/w respectively .Further investigation were carried out for In-vitro antioxidantactivity and Radical scavenging assay by calculating its %inhibition by means of IC_{50} values (it is nothing but inhibition concentration to obtain 50% of maximum scavenging capacity),all the extracts concentration has been adjusted to come under the linearity range and here many reference standards like Tannic acid, Gallic acid, Quercetin, Ascorbic acid have been taken for the method suitability. The results revealed that the leaves of this plant have antioxidant potential .Among these results ethanolic extract has more potent than traditionally claiming aqueous decoction. In conclusion that *Lagenaria siceraria* leaves possesses the antioxidant substance which may be potential responsible for the treatment of jaundice and other oxidative stress related diseases.

Keywords: *Lagenaria siceraria* leaves (L.S), Total Phenolic Content (TPC), Ferric reducing antioxidant power (FRAP), radical scavenging assay (DPPH – RSA)..

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INTRODUCTION

Lagenaria siceraria commonly known as Bottle gourd called as Doodhi in Gujarati, Lauki (Hindi), Kadoo (Marathi) which is official in Ayurvedic Pharmacopoeia. It is one of the excellent fruit for human being made and gifted by the nature having composition of all the essential constituents that are required for normal and good human health(1). The archaeological evidences suggest that *Lagenaria* is not a monotypic genus and has an ancient pan tropical distribution. Two varieties of this fruit drug sweet and bitter are mentioned. Botanically, both belongs to the same genus, the sweet varieties is generally used as a vegetable, while the wild Variety bitter, preferred for the medicinal use. The difficulty in procuring and loosing interest in cultivation of wild variety, the sweet and edible variety is now being used in medicine as well(2). Leaves of *Lagenaria siceraria* are taken as emetic in the form of leaf juice or decoction. This by adding sugar also used in Jaundice. Crushed leaves are used for baldness and applied on the head for the headache. Leaves are also used as alternative purgative (3-4). The edible portion of fruits is fair source of ascorbic acid, beta carotene and good source of vitamin B complex, pectin dietary soluble fibers and contains highest source of choline level-anisotropic factor, a healer of mental disorders, along with required metabolic and metabolite precursors for brain function, amongst any other vegetable known to man till date. It is also good source of minerals and amino acids (5-6). The fruit is reported to contain the triterepeniode cucurbitacins B, D, G, H and 22-deoxycucurbitacin “the bitter principle of cucurbitaceae”. The fruit juice contains

betaglycosidedase-elasterase enzyme. Two sterols were identified and isolated from petroleum ether fractions of ethanol extract of dried fruit pulp of L.S namely Fucosterol and campesterol(7). HPLC analysis of extract of flowering plant of *Lagenaria siceraria* shows presence of flavone-C glycosides (8).The effect of semi purified dietary fibers isolated from the fruit of L.Seffects on fecal steroid excretion was reported(9). Although the L.Splant leaves carries high potential uses, especially for the treatment of jaundice, but the proper scientific studies have not been much reported for the leaves of this plant especially for an antioxidant perspective, an antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols(10). As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease. Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials did not detect any benefit and suggested instead that excess supplementation may be harmful. In addition to these uses of natural antioxidants in medicine, these compounds have many industrial uses, such as preservatives in food and cosmetics (11). So that we got an interest to fulfill the paucity of studies by means of preliminary In-vitro antioxidant work which we have carried out in leaves portion of *Lagenaria siceraria*, here is our initiation for the future drug.

MATERIAL AND METHODS

Chemicals and reagents

Chemicals used in this study were 1, 1-diphenyl-2-picrylhydrazyl (DPPH) obtained from Sigma-Aldrich, U.S.A, Phosphomolybdic acid, sodium tungustate, potassium ferricyanide and sodium nitroprusside, naphthylethylenediamine dihydrochloride, sodium nitrite, trichloroacetic acid, ascorbic acid, ethylenediamine tetraacetic acid, phosphoric acid, nitro bluetetrazolium, phenazine methosulfate, ferrous ammonium sulfate are obtained from Sd Fine Chemicals Ltd, India. All other reagents and solvents used in the study were of analytical grade.

Plant material

The leaves of *Lagenaria siceraria* (Family: cucurbitaceae) were collected with flowering top during the month of October from local cultivating field area of Mehsana district, Gujarat, India. The plant material was authenticated at the Department of Botany, Govt. Arts and science College Himatnagar, Gujarat. A voucher specimen as a herbarium (LS/RES/SSPC-05/2007) has been kept in our museum for future reference. The leaf parts were chopped and dried at room temperature for 10 days and used as raw material. The dried leaves of the drug have powdered using mechanical method and resulting powder was passed through the 40 # sieve and stored in the airtight container.

PREPARATION OF RAW MATERIAL

Preparation of crude aqueous extract

Then weighed accurately 100 gm of powder was taken in stainless steel vessel and mixed with 2000 ml (1:20) of distilled water. Then the mixture was boiled for about 2 hours using gas burner. After that, the mixture was filtered through cotton bag and then using vacuum filter assembly (or) fixed standard Whatman filter paper size No.1, here the filtrate must be poured in a borosilicate 500ml beaker. Then the filtrate was evaporated on hot plate until it reaches the concentrated quantity (do not be in viscous state)

To prepare dry powder form of extract

The dry powder of this extract was prepared by using the simple saloon water sprayer by spraying the extract on stainless steel evaporating plate, after the predetermined flow conditioned consistency thick solution was poured into the sprayer (here the above concentrated extract solution varies to nature of plant material), by which it was heated on hot plate at constant temperature of about 60°C. The clumpy dry powder obtained was scraped by the knife and made into fine powder form by using preheated mortal and pestle glass type from the plate and packed in air tight plastic container every steps must be carried out at above the room temperature (demerit of this method can be overcome by using hot air hair dryer) and stored in the freezer or in vacuum desiccator's as such or in the form of stock solution prepared by the same solvent until further use. The preconditioned set method can be optimized by

evaluating the quantitative test of any existed constituents like tannins, flavonoids or any existed markers by suitable validated methods. This present study was undertaken by the spectrophotometrical method

Preparation of ethanolic extracts

Then weighed loading limit amount of 45.20 gm of powder of drug was packed in thimble flask and 550ml of ethanol (70%) was added in 1 liter round bottom flask. Then the Soxhlet assembly was set up to complete 10 to 15 cycles. After that the extract was filtered and filtrate was concentrated up to 50 ml using water bath. From the concentrated 10ml of extract was taken in evaporating dish (Borosilicate glass) which is previously weighed. The total weigh of evaporating dish containing 10ml extract was recorded and the extract was evaporated till thick liquor was obtained. After then calculate the difference in weight was noted at every 10 min until the constant weigh was obtained. The residue at the constant weigh (it can be obtained from the graph %L.O.D) is used as dry extractives (12), which can be used to prepare the stock solutions (w/v). The weight of dry extract was 0.94 gm and the total yield was (4.5 gm) and the % yield would be 9.96% w/w and stored in the freezer until further use

QUALITATIVE PHYTOCHEMICAL SCREENING

The shade dried leaves was coarsely powdered and extracted with different solvents like n-Hexane, Chloroform, Ethyl acetate, 70% Ethanol and Water successively. All the extracts were concentrated under reduced temperature and pressure to get dry residue. The different qualitative chemical tests were performed (13) for establishing profile of extracts and to detect the various phytoconstituents present in them. But the alcoholic and aqueous extracts showed much of the antioxidant potential constituents (14). So that our studies as carried out the extracts made by using these solvents only.

ESTIMATION OF TPC BY SPECTROPHOTOMETER

By Folin – Denis Method

The method is based on the oxidation of molecule containing a –OH groups. The tannin and tannin like compound reduce Phosphotungustomolybdic acid in alkaline solution to produce a highly blue colored solution (15-16). 1ml of the aqueous and ethanolic extract that has adjusted to come under the linearity range i.e. (50µg/ml) of both the drugs was withdrawn in 10ml volumetric flask separately. To each flask 0.5ml of Folin-Denis reagent and 1ml of Sodium carbonate was added and volume is made up to 10ml with distill water. The absorbance was measured at absorption maxima 700nm within 30 minute of reaction against the blank. The total phenolic content was determined by using calibration curve (5 to 30µg/ml).Three readings were taken for each and every solution for checking the reproducibility and to get accurate result. Results are provided in (Table 2 and Figure 1).The intensity of the solution is proportional to the amount of tannins and can be estimated against standard tannic acid, the total phenolic content, expressed as mg tannic acid equivalents per 100 g dry weight of sample.

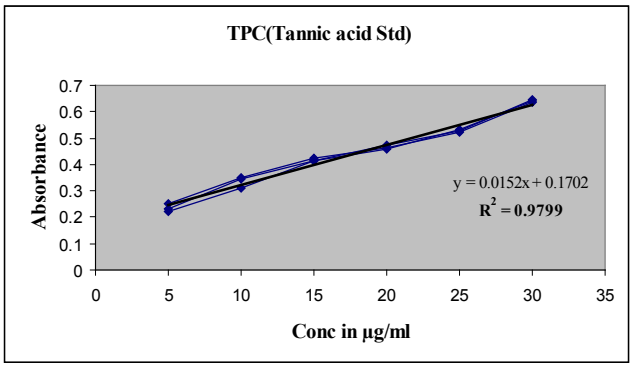

Figure 1: Results of Totalphenolic content.
R^2 values represented mean data set of n+3

Table 2: Results of Totalphenolic content

No	conc. of extracts	%w/w of total tannin
1	L.S Aqueous. 50µg/ml	13.75 ± 0.22
2	L.S Ethanolic. 50µg/ml	22.12 ± 1.23

Values are mean ± S.E.M, n=3

TOTAL FLAVONOID CONTENT BY SPECTROPHOTOMETER

Aluminum chloride colorimetric assay method

Total flavonoid contents were measured with the aluminum chloride colorimetric assay. (17) .Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distill water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Results are provided in (Table 3 and Figure 2).Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

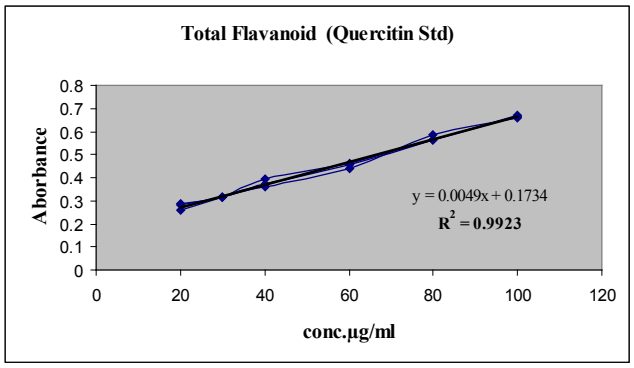

Figure 2: Results of Flavonoid content.
R^2 values represented mean data set of n+3

Table 3: Results of Flavonoid content

No	conc. of extracts	%w/w of total Flavonoid
1	L.S Aqueous. 400µg/ml	1.33 ± 0.08
2	L.S Ethanolic. 400µg/ml	3.61 ± 0.32

Values are mean ± S.E.M, n=3

IN-VITRO ANTIOXIDANT STUDY

FRAP method

The ferric reducing property of the extract was determined by (18) taking 1ml of different dilutions of standard solutions of Gallic acid (10 -100 µg/ml) or aqueous and ethanolic extract that has adjusted to come under the linearity range (500µg/ml) was taken in 10ml volumetric flasks and mixed with 2.5ml of potassium buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20min. Then 2.5ml of ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. To the 2.5ml of above solution 2.5ml of distill water is added and then 0.5ml of 0.1% of $FeCl_3$ was added and allowed to stand for 30min before measuring the absorbance at 593 nm. Results are provided in (Table 4 and Figure 3).The absorbance obtained was converted

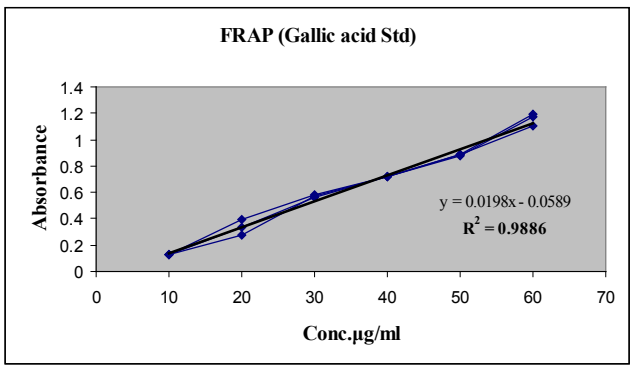

Figure 3: Results of FRAP
R^2 values represented mean data set of n+3

Table 4: Results of FRAP

No	Conc. of extracts	mg GAE/gm of extracts
1	L.S Aqueous. 500µg/ml	53.76 ± 0.28
2	L.S Ethanolic. 500µg/ml	66.53 ± 2.54

Values are mean ± S.E.M, n=3

to Gallic acid equivalent in mg per gm of dry material (GAE/gm) using Gallic acid standard curve.

SCAVENGING ACTIVITY ASSAYS

Nitric oxide scavenging assay

Nitric oxide radical inhibition was estimated by the use of Griess Illosvory reaction (19-20).In this investigation, Griess Illosvory reagent was generally modified by using Napthyl ethylene diamine dihydrochloride (0.1%w/v) instead of the use of 1-napthylamine (5%). The reaction mixture (3ml) containing 2ml of 10 mM sodium nitroprusside, 0.5ml saline phosphate buffer and 0.5ml of standard solution or aqueous and ethanolic extract of (500 -1000µg/ml) were incubated at 25°C for 150min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml Sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5min for the completion of the reaction of diazotization. After that further 1ml of the Napthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30min at 25°C. The concentration of nitrite was assayed at 546nm and was calculated with the control absorbance of the standard nitrite solution (without extracts or standards, but the same condition should be followed).Here the blank is taken as the buffer and make up solvents and the Ascorbic acid and Quercetin (10 -50 µg/ml) was taken as standard. Results are provided in (Figure 4–7) .The percentage inhibition was calculated using the formula:

$$\%\text{Scavenging Activity} = \frac{\text{A control A test or A Std}}{\text{A control}} \times 100$$

Where, $A_{control}$ + absorbance of control
A_{test} or A_{Std} + absorbance of test or std

Hydrogen Peroxide scavenging Assay

The ability of extracts to scavenge hydrogen peroxide was determined (21) by little modification here the solution of hydrogen peroxide (30mm) was prepared instead of 40mM in phosphate buffer saline of (PH 7.4), at various concentration of aqueous and ethanolic extract (100 -1000 µg/ml) were added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was

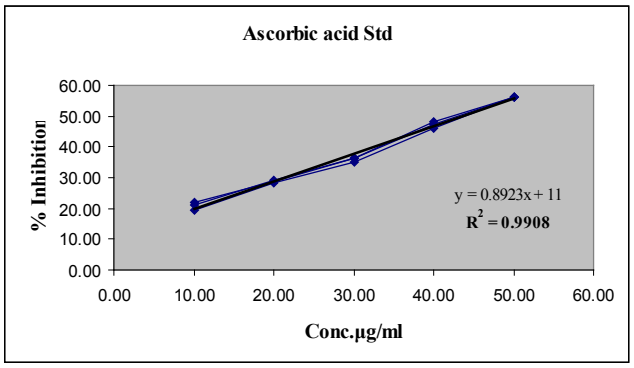

Figure 4: Results of Nitric oxide radical scavenging assay. R^2 values represented mean data set of n+3

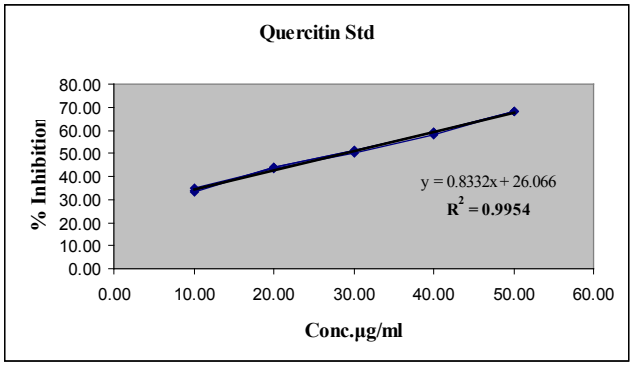

Figure 5: Results of Nitric oxide radical scavenging assay R^2 values represented mean data set of n+3

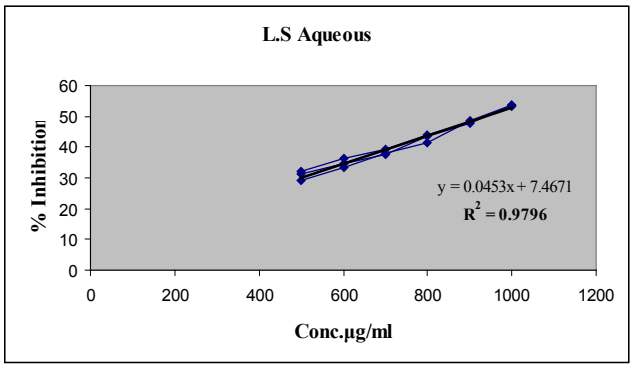

Figure 6: Results of Nitric oxide radical scavenging assay R^2 values represented mean data set of n+3

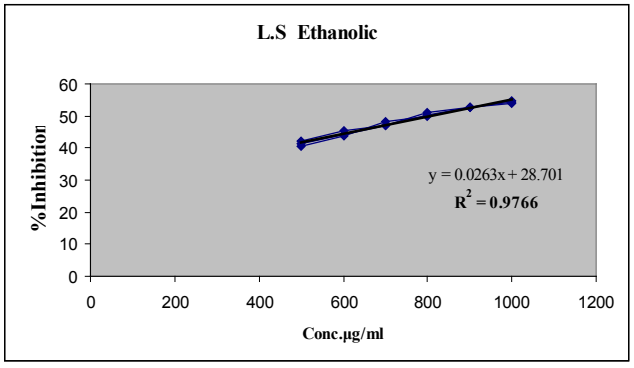

Figure 7: Results of Nitric oxide radical scavenging assay R^2 values represented mean data set of n+3

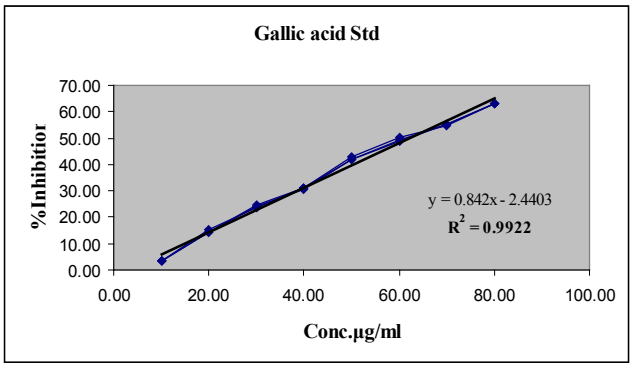

Figure 8: Results of Hydrogen Peroxide scavenging Assay R^2 values represented mean data set of n+3

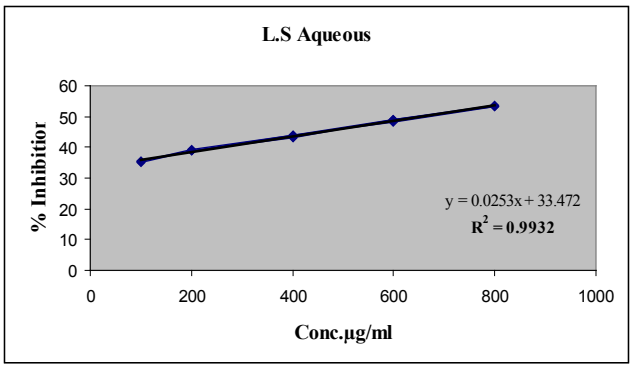

Figure 9: Results of Hydrogen Peroxide scavenging AssayR^2 values represented mean data set of n+3

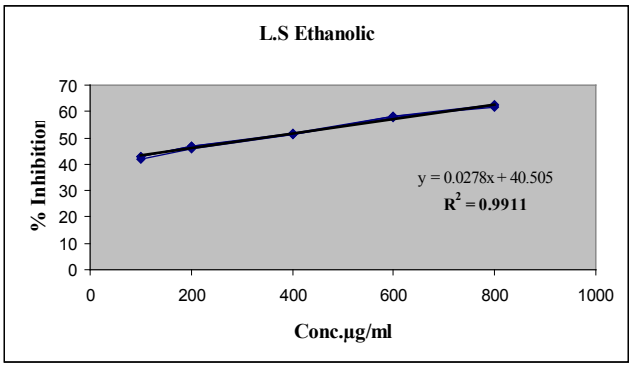

Figure 10: Results of Hydrogen Peroxide scavenging Assay R^2 values represented mean data set of n+3

determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for back ground subtraction. In case of control takes absorbance of hydrogen peroxide at 230 nm without sample extracts. Results are provided in (Figure 8–10).The percentage inhibition activity was calculated from $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of extract/standard taken as Gallic acid (10 -100 µg/ml).

DPPH –RSA method

The free radical scavenging activity of aqueous and ethanolic extracts and the standard L-Ascorbic Acid (Vitamin C) was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (22-23)here,0.1mM solution of DPPH in alcohol was prepared and it must be protected from light influence by maintaining the dark condition and also fold by aluminum foil and 3ml of this solution was added to 1ml various conc.(100-2000 µg/ml) of extracts or standard solution of (10-100 µg/ml). Absorbance was taken after 30min at 517nm. Results are provided in (Figure 11–13).

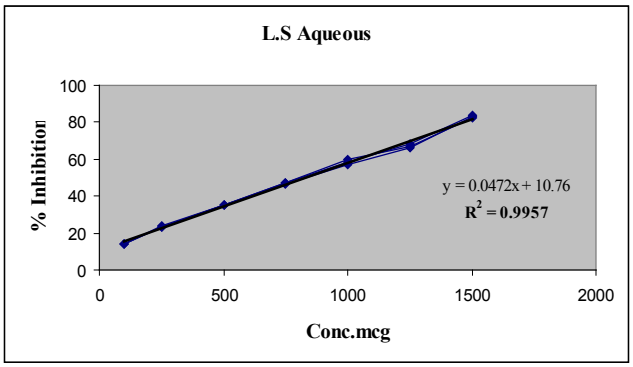

Figure 12: Results of DPPH radical scavenging assay
R^2 values represented mean data set of n+3

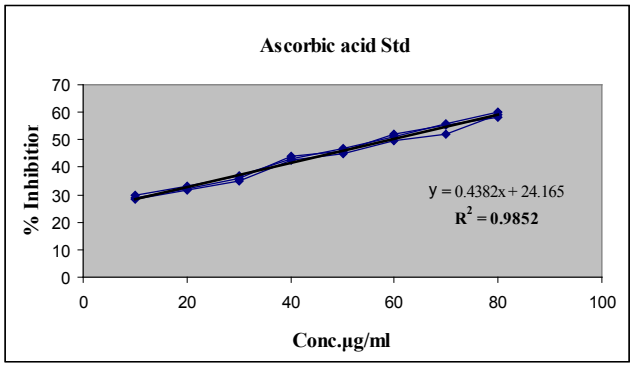

Figure 11: Results of DPPH radical scavenging assay
R^2 values represented mean data set of n+3

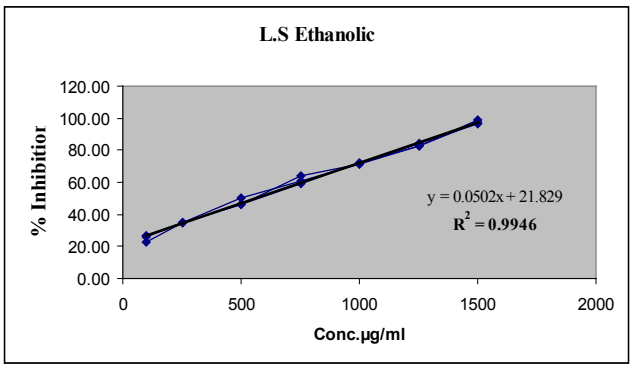

Figure 13: Results of DPPH radical scavenging assay
R^2 values represented mean data set of n+3

Table 1: Results of Qualitative Phytochemical screening:

Tests	n-Hexane	Chloroform	Ethyl acetate	70%ethanol	Water
Carbohydrates	–	–	–	+	+
Alkaloids	–	–	–	–	–
Glycosides	–	–	–	+	+
Steroids	++	+	+	+	–
Tannins	–	–	–	++	+
Flavonoids	–	–	–	++	+

Indicators:

+: Positive; ++: More Positive (intensity of color or ppt)

–: Negative

The percentage inhibition activity was calculated from $[(A_0-A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of extract/standard taken as Ascorbic acid.

STATISTICAL ANALYSIS

Values were represented as mean ±S.E.M of three parallel data's.

RESULTS AND DISCUSSION:

Results of Qualitative Phytochemical screening

The qualitative analysis of the extracts was carried out and the results obtained were given in (Table 1). The results revealed that the presence of carbohydrates, glycosides, phytosterols, tannins and phenolic compounds, flavonoids.

Effect of TPC & Flavonoid content

The quantitative determination of the total phenolic content, expressed as mg tannic acid equivalents and per 100 g dry weight of sample TPC of L.S aqueous and ethanolic extracts showed the content values of 13.75± 0.22%w/w and 22.12± 1.23%w/w and total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample the total flavonoids estimation of aqueous and ethanolic extracts showed the content values of 1.33 ± 0.08%w/w and 3.61 ± 0.32%w/w.The above results showed that aqueous contain less tannins and flavonoid content than the alcoholic extract. It may due to the solubility of principle contents presence be higher incase of alcoholic solvent, thus it has been accepted that it is a universal solvent for the extraction of plant constituents.

Capacity of FRAP method

At low pH, measuring the change in absorption at 593 nm can monitor reduction of a ferric complex to the ferrous form, which has an intense bluish green color. The change in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. Here the FRAP showed the results of aqueous and ethanolic extracts that of 53.76 ± 0.28 mg equivalent to Gallic acid(GAE)/gm of sample and 66.53 ± 2.54 mg GAE/gm of sample respectively.

Capacity of Nitric oxide scavenging assay

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O_2 to produce the stable product nitrates and nitrite through intermediates through NO_2, N_2O_4 and N_3O_4. It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. The extent of decrease will reflect the extent of scavenging, the %inhibition of aqueous and ethanolic extract of three parallel readings of (r^2+0.9796) showed that IC_{50} values938.92µg/ml and 805.85µg/ml (r^2 + 0.9766) respectively as compared to the standard of Ascorbic acid and Quercetin of 43..71µg/ml (r^2 + 0.9908) and 28.73µg/ml (r^2 + 0.9954) respectively.

Capacity of Hydrogen Peroxide scavenging

H_2O_2 itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells. Thus, removal of H_2O_2 is very important for protection of food systems. Scavenging of Hydrogen per oxide and its %inhibition of aqueous and ethanolic extract showed that IC_{50} values 653.28µg/ml (r^2 + 0.9932) and 341.55µg/ml (r^2 + 0.9911) respectively. Gallic acid has taken as reference which showed 62.28µg/ml. (r^2 + 0.9922)

Capacity of DPPH –RSA

The antioxidant reacts with stable free radical, DPPH and converts it to 1, 1- Diphenyl-2- Picryl Hydrazine. The ability to scavenge the free radical, DPPH was measured at an absorbance of 517 nm. So the DPPH – RSA and its %inhibition of aqueous and ethanolic extract showed that IC_{50} values 831.36µg/ml (r^2 + 0.9957) and 561.18µg/ml (r^2 + 0.9946) respectively. Ascorbic acid has taken as reference which showed 58.96µg/ml. (r^2 + 0.9852) among these results ethanolic extract has more potent than traditionally claiming decoction.

The overall results of % inhibition as shown in the (Table 5) respective to IC_{50} values and regression r^2 is the mean value of (n+3).

Table 5: Results and discussion of all the % Inhibition studies

Results of % Inhibition	No.	Samples	*r^2 values	I_c 50 values
% Inhibition by Nitric	1	Standard Ascorbic acid	0.9908	43.71µg/ml
oxide assay	2	Standard Quercitin	0.9954	28.73µg/ml
	3	L.S Aqueous	0.9796	938.92µg/ml
	4	L.S Ethanolic	0.9766	805.85µg/ml
% Inhibition by Hydrogen	1	Standard Gallic acid	0.9922	62.28µg/ml
Peroxide assay	2	L.S Aqueous	0.9932	653.28µg/ml
	3	L.S Ethanolic	0.9911	341.55µg/ml
% Inhibition by DPPH-RSA	1	Standard Ascorbic acid	0.9852	58.96µg/ml
	2	L.S Aqueous	0.9957	831.36µg/ml
	3	L.S Ethanolic	0.9946	561.18µg/ml

*Data set of n=3 and mean r^2 values obtained from the graphs.

CONCLUSION

In conclusion that *Lagenaria siceraria* leaves possesses the antioxidant substance which may be potential responsible for the treatment of jaundice. So there are many scopes are there in leaves portion and more number of studies can be undertaken like oxidative stress hepatoprotective, anticancer activities and etc. In future we look forward to check the potency of the leaves by means of In-vivo antioxidant studies.

ACKNOWLEDGMENT

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REFERENCES:

1. Habibur Rahaman AS. Bottle gourd (Lagenaria siceraria) - a vegetable for good health. *Natural Product Radiance*. **2** (5):249–256(2003).
2. Sivarajan SS, Balchandra A. *Ayurvedic Drugs and their Plant Source*,(Oxford and IBH, Publication Company Pvt. Ltd., New Delhi, 2003)176–177
3. Nadkarni. *Indian MatericaMedica*,Vol-**1**,(Popular Prakashan Pvt.Ltd, 1992)722–723
4. Chopra BN, Chopra IC. *Glossary of Indian Medicinal Plants*, (Publication and Information Directorate, CSIR, Council of Scientific and Industrial Research, New Delhi, India, 1992)148
5. Anonymous. *Wealth of India(Raw materials)*, Vol-**V1**,(Council of Science and Industrial Research, New Delhi: Publication and Information Directorate, 1996)16–19
6. Kirtikar KR, Basu BD, *Indian Medicinal Plants*, 2ed,Vol-**IV**(Oriental Enterprises, Dehradun,2001)
7. Shirwaikar A, Sreenivasan KK. Chemical investigation and antihepatotoxic activity of the fruits of Lagenaria siceraria.*Ind.J.Pharm.Sci*. **58** (5):197–202(1996).
8. Baoranoswka KM, Cisowski W.HPLC determination of flavone-C glycosides in some species of Cucurbitaceae family, *J Chromatogram-A*. **675**:240–243 (1994).
9. Sannoumaru YJ, Shimizu. Effects of semi-purified dietary fibers isolated from Lagenaria siceraria, Raphanus sativus and Lentinus edobuson fecal steroid excretion in rats. *J. Nut.Sci.Vitamin*. **42**(2):97–110 (1996).
10. Hyoung Lee S. Antioxidant activity of browing reaction product isolated from storage-aged orange juice. *J Agric Food Chem*. **40** (4):550–552 (1992).
11. Garrat DC. *The quantitative analysis of drugs*, Vol-**III** (Chapman and Hall Ltd., Japan, 1964).
12. *The Ayurvedic Pharmacopoeia of India* 1st edition, 233–240 (1990).
13. Raman N, *Phytochemical techniques* (New India publishing agency New Delhi 2006)19–24.
14. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol*.**32**:1141–1148 (1983).
15. Cetkovic G, Canadanovic J, et al., Assessment of Polyphenolic content and in vitro antiradical characteristics of apple pomace, *Food Chemistry*.**109**:340–347 (2008).
16. Jain U.K. and Dixit V.K spectrophotometrical estimation of tannins from the Chyavanprash, *Journal of Indian drugs*. **41**:469–472 (2004).
17. Kumar S. et al., Antioxidant free radical scavenging potential of Citrullus colocynthis (L.) Schrad. Methanolic fruit extract, *Acta pharma*.**58**:215–220(2008).
18. Lim Y.Y., Murtijaya J.*LWT*.**40**:1664–1669 (2007).
19. Chakraborthy G.S., Free radical scavenging activity of Costus speciosus leaves, *Indian Journal of Pharmaceutical Education and Research*.**43**(1):96–98 (2009).
20. Hyoung Lee S., Antioxidant activity of browing reaction products isolated from storage aged orange juice, *J agric Food Chem*. **40**(4): 550–552 (1992).
21. Gülçin I., Alici H. A. and Cesur M., Determination of in vitro antioxidant radical scavenging activities of propofol *Chem, Pharm. Bull*. **53**:281–285 (2005).
22. Sreejayan and Rao M. N. A., Free radical scavenging activity of curcuminoids. *Drug Res*. **46**:169–171 (1996).
23. Mohammad T.A., Determination of Polyphenolic content and free radical scavenging activity of Flemingia strobilifera, *Advances in Pharmacology and Toxicology*. **10** (1):89–95 (2009).

Anti-nociceptive and anti-inflammatory activities of *Atalantia retusa* Merr.

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Abstract

Atalantia retusa is an endemic medicinal plant used in the Philippines. Hexane extract from leaves were orally administered on rats and mice and tested using rat paw edema, formalin and writhing assays. Increased pain tolerance was observed in animals administered with the median dose (1.43 mg/Kg BW) in both somatic ($P<0.05$) and visceral ($P<0.01$) models by 6.05% and 55.48% better compared to the positive control. The degree of swelling was also reduced by the administration of 1.43 mg/Kg BW at 0.5 to 3.0h after carrageenan injection suggesting a high impact analgesic and anti-inflammatory effects of *A. retusa* hexane extract.

Keywords: *Analgesic, Anti-inflammatory, Rutaceae, hexane extract..*

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1. INTRODUCTION

Since the earliest times, man has turned to nature for remedies to alleviate pain. Traditional folk medicine usually involves a variety of indigenous plants and herbs for particular illnesses. The Philippines has a rich cultural background complimented with a diverse range of flora. Traditional folk medicine therefore is a common practice in rural areas for people seeking remedy from *herbolaryos* (folk medicine man) for treatment of various illnesses such as toothache, fever, stomach ache, cramps and the like. This has been the basis for the discovery of bioactive compounds that are now being used as prescription drugs.

Pain is usually perceived throughout the body by the numerous specific nociceptors. Analgesic drug relieves pain by blocking pain signals going to the brain or interfering with the brain's interpretation of signals [1]. Cyclooxygenase inhibitory (COX) drugs inhibit COX-2 thus preventing the convertion of arachidonic acid into prostaglandins formed when membrane bound phospholipids come into contact with phospholipase A2 in the event of trauma or inflammation of tissue [2]. Reduced levels of prostaglandin will also result in the reduction of cytokines such as interleukin-2 (IL-2) thereby desensitizing the CNS and PNS. The desensitized integrating centers therefore will have less sensitivity to pain.

Information on *Atalantia retusa* locally known as *tulan manok* is very limited, although, other species of the Rutaceae family have been studied for its anti-inflammatory, analgesic, antispasmodic and antinociceptive properties. *A. retusa* is a tree growing to a height of about 4-5 meters at an altitude of 130m ASL. It is an endemic species inhabiting the non-teak forests of Mindoro, Palawan, and Panay islands in the Philippines. Locals in Occidental Mindoro claim that the plant is used to treat various ailments among locals. Other members of the Rutaceae family have been documented to have significant antinociceptive and anti-inflammatory properties, but there are no documented studies confirming the antinociceptive and anti-inflammatory properties of *A. retusa*. The current study sought to verify such potential of *A. retusa*.

2. EXPERIMENTAL

2.1 Collection of Plant Material

Atalantia retusa leaves from the non-teak forest of Sitio Bunlao, Baranggay Ipil, Ilin Island, San Jose, Occidental Mindoro was collected in April, 2008 and identified by Dr. Emelina H. Mandia with voucher number 893 deposited at the Biology Department, DLSU-Manila.

2.2 Preparation of Plant Extracts

The air-dried leaves (800 grams) were first pulverized and then soaked in 2L Hexane (Ajax FineChem, Australia) for 3 days and then filtered. The filtrate was then concentrated under vacuum to afford 4.47g of non-polar extract. The extract was further liberated from the extracting solvent by desiccation. Polysorbate80 (25%) in corn oil was used as vehicle and kept in cold storage until time of use.

2.3 Animals

Laboratory-bred male ICR Mice (*Mus musculus* L.) and Sprague-Dawley Rats (*Rattus norvegicus*) from parent stocks obtained from the Experimental Animal House of the Bureau of Food and Drugs, Muntinlupa City, Philippines were used in the study. A total of 45 male (6-week old) mice, and 70 male (6-week old) rats weighing an average of 30.44±3.74g and 197.03±26.17g, respectively, were acclimatized for 14 days followed by a 2 hour post-acclimatization before the assay proper. The animals were kept in the Animal Containment unit of De La Salle University, under normal conditions with 12 hours daylight and 12 hours darkness, with free access to food pellets (28 %CP, 14% CF) and water. All procedures regarding handling of the test animals were in accordance with the existing guidelines of the Philippine Association of Laboratory Animal Science (PALAS) for care and use of laboratory animals [3] and with Administrative Order 40 of the Bureau of Animal Industry relative to Republic Act No. 8485.

2.4 Antinociceptive activity

2.4.1 Formalin Test [4]

Inhibition of somatic pain was tested on rats (n=6) orally administered with increasing dosages of *A. retusa* non-polar extract (0.143mg/kg BW, 1.43mg/kg BW and 14.3mg/kg BW) followed by injection of 1% Formalin on the right hind paw 1 hour after the administration of the test drug. Paracetamol (10 mg/kg BW, Bristol-Meyers) and 25% Polysorbate80 (25%) were used as positive and negative controls, respectively. The number of bites and paw licks were counted for 10 minutes followed by a 30-minute interval before the second counting period. Total number of scratches and licks are presented as % inhibition. Percent inhibition is computed as 100 – [(Number of bites or scratches per individual/average number of bites or scratches of the negative control) × 100]

2.4.2 Acetic Acid test [5]

Inhibition of visceral pain was tested on mice (n=9) orally administered with Polysorbate80 (25%), Paracetamol (10mg/kg BW), and the non-polar extract of *Atalantia retusa*, in 3 different dosages followed by an intraperitoneal injection of 1% Glacial Acetic Acid after 1 hour. The number of abdominal stretches completed within 10 minutes were counted and presented as % inhibition.

2.5 Anti-inflammatory activity [6,7]

*M*ale Sprague-Dawley rats (n=8) were orally administered with either nonpolar extract (0.143, 1.43 and 14.3 mg//Kg BW), diclofenac sodium difenax, (1.43 mg/Kg BW, GX International, Philippines) or vehicle (1 ml/ Kg BW) followed by a single plantar injection of 0.1 ml 1% λ-carrageenan (Sigma) at the right foot 1h after oral gavage. The contra-lateral foot was injected with 0.1 ml physiological saline (0.9% NSS) as control. Paw volume was measured plethysmographically before and 0.5, 1, 1.5, 2, 2.5 and 3h after carrageenan injection. The degree of swelling was determined by obtaining the ratio of a/b where *a* and *b* are volumes of the same hind paw after and before carrageenan treatment, respectively. A ratio that is smaller than 1.25 indicates a significant inhibitory effect of the extract.

2.7 Statistical Analysis

The results were analyzed using SPSS ver. 13 for Windows. One way Analysis of Variance was performed to determine the significant effects of the analgesic potentials of the *A. retusa* non-polar extracts. The results were considered significant at $P \leq 0.05$. Significant differences between group variables were determined by post hoc analysis at 95% DMRT. Means represent Mean ± SD.

3. RESULTS AND DISCUSSION

3.1 General Observation

The test animals did not show observable indicators of intoxication nor have yielded incidence of mortality brought about by the effects of the extract. This indicates that the dose of the extract tested is non-toxic to the animal which is further confirmed by zero mortality after 24 hours.

3.2 Antinociceptive activity

3.2.1 Formalin test

Male Sprague-Dawley rats received an oral dose of *A. retusa* non-polar extract, followed by a single injection of 1% formalin to the right hind paw 1 hour after administration of the test drug. The number of paw licks was counted for 10 minutes, followed by a second counting at 30 minutes

interval. The lowest concentration (0.1437mg/kgBW) and medium concentration (1.437mg/kgBW) of the non-polar extract have shown significant analgesic properties comparable to the effects of Diclofenac (15.67±4.47) evident in the reduced paw licking and biting compared to the negative control (27.00±6.27). The highest concentration of *A. retusa* extract has shown very little analgesic activity (Table 1).

3.2.2 Acetic Acid Writhing Assay

Male ICR mice orally administered with increasing dosages of *A. retusa* non-polar extract were given a single injection of 1% Glacial Acetic Acid 1 hour after the administration of the test drug. The number of abdominal stretching was immediately counted for 10 minutes after injection of glacial acetic acid. *A. retusa* non-polar extract have shown significant analgesic effect (P<0.001) at 1.43mg/kg BW and 14.3mg/kg BW which is non comparable with those given the positive control. Mice administered with the median dose of the non-polar extract obtained 90.51±10.05% compared to those mice given with Paracetamol 15.67±4.47%, further the non-polar extract was able to surpass the inhibitory effect of Paracetamol by 74.48%. The lowest concentration however (0.0143mg/kg BW) revealed no antinociceptive effect which is not significantly different with the negative control (-0.1±55.46%). (Tab. 1)

3.3 Anti-inflammatory activity

Atalantia retusa nonpolar extract has significantly reduced inflammatory reaction in λ-carrageenan injected paw. The degree of paw swelling in rats treated with the median dose (1.43 mg/Kg BW) was effectively (P<0.022) reduced by 1.043±0.26 which is not significantly different with the anti-inflammatory activity of Diclofenac (1.08±0.17) at a dose similar to the median dose treatment 1.0h after λ-carrageenan injection. The inhibitory effect of the median dose persisted until 3.0h (Tab. 2) which is still not significantly different with diclofenac. This indicates the positive control and the extract had comparable activity at the dose level tested. The inhibition of inflammation, however and was more

Table 1 Somatic and Visceral Pain Models

	Somatic Model (Formalin Test)		Visceral Model (Writhing Test)	
	No. of Paw Licks	**% Inhibition**	**Number of Writhes**	**% Inhibition**
Paracetamol	15.67±4.41^{b}	28.80±20.05^{a}	19.78±10.20^{b}	35.03 ± 33.50^{b}
25% Polysorbate80	22.00 ± 6.26^{a}	0.0 ± 28.46^{b}	30.44 ± 16.88^{a}	–0.15 ± 55.46^{c}
0.1437 mg/kg BW	15.5 ± 4.09^{b}	29.54. ± 18.57^{a}	31.22 ± 4.06^{a}	–2.57 ± 13.32^{c}
1.437 mg/kg BW	14.33 ± 3.14^{b}	34.85 ± 14.28^{a}	2.89 ± 3.06^{c}	90.51 ± 10.05^{a}
14.37 mg/kg BW	18.67 ± 3.78^{ab}	15.15 ± 17.17^{ab}	6.78 ± 6.30^{c}	77.73 ± 20.70^{a}

**Means followed by the same letter is not significantly different at 95% DMRT.(0.05α)*

Table 2 Degree of swelling in rat hind paw.

	Right Paw (λ-carrageenan)					
	0.5h	**1.0h**	**1.5h**	**2.0h**	**2.5h**	**3.0h**
25% Polysorbate80	1.36 ± 0.38	1.33 ± 0.35^{abc}	1.52 ± 0.39^{ab}	1.54 ± 0.33^{ab}	1.48 ± 0.34^{ab}	1.56 ± 0.45^{b}
Diclofenac	1.14 ± 0.13	1.08 ± 0.17^{ab}	1.27 ± 0.30^{a}	1.31 ± 0.19^{a}	1.23 ± 0.02^{a}	1.14 ± 0.13^{a}
0.143 mg/kg BW	1.28 ± 0.28	1.37 ± 0.3^{bc}	1.64 ± 0.36^{b}	1.59 ± 0.24^{ab}	1.67 ± 0.36^{b}	1.59 ± 0.45^{b}
1.43 mg/kg BW	1.06 ± 0.19	1.04 ± 0.26^{a}	1.23 ± 0.32^{a}	1.31 ± 0.33^{a}	1.26 ± 0.39^{a}	1.20 ± 0.40^{ab}
14.37 mg/kg BW	1.12 ± 0.17	1.43 ± 0.23^{c}	1.68 ± 0.22^{b}	1.72 ± 0.33^{b}	1.68 ± 0.22^{b}	1.43 ± 0.18^{ab}
	Left Paw (0.9% Saline)					
25% Polysorbate80	1.08 ± 0.17^{ab}	1.18 ± 0.20	1.25 ± 0.18	1.25 ± 0.13	1.21 ± 0.18	1.18 ± 0.14
Diclofenac	1.21 ± 0.16^{b}	1.01 ± 0.12	1.22 ± 0.28	1.23 ± 0.26	1.29 ± 0.21	1.19 ± 0.27
0.1437mg/kg BW	1.23 ± 0.23^{b}	1.12 ± 0.17	1.29 ± 0.23	1.23 ± 0.26	1.39 ± 0.35	1.16 ± 0.25
1.437mg/kg BW	0.98 ± 0.06^{a}	1.01 ± 0.11	1.00 ± 0.17	1.16 ± 0.10	1.15 ± 0.25	1.02 ± 0.17
14.37mg/kg BW	0.94 ± 0.17^{a}	1.09 ± 0.13	1.14 ± 0.20	1.20 ± 0.18	1.13 ± 0.19	1.21 ± 0.26

**Means followed by the same letter is not significantly different at 95% DMRT.(0.05α)*

pronounced during the early phase of inflammatory reaction suggesting the central neurologic action of the extract. Injection of physiological saline in the left paw indicates that the subplantar injection had minimal involvement in the inflammatory reaction in the test animals confirming that the inflammation was due to carrageenan and the inhibition of inflammation was due to the activity of the extract. The lowest dose (0.143 mg/Kg BW) revealed no inhibition of paw swelling particularly suggesting that the dose is too low to demonstrate pharmacologic effect while the highest dose (14.3 mg/Kg BW) is too high that the maximum effective dose has been reached that no obvious pharmacologic effect is observed.

3.4 Discussion

Overall, the non-polar extract from *A. retusa* leaves has shown significant analgesic effect, which has been observed to be more effective than paracetamol or diclofenac. The complementary action of analgesic and the anti-inflammatory activity in known test drugs such as paracetamol and diclofenac was significantly demonstrated in the experimental animals treated with median dose *A. retusa* nonpolar extract with no significant differences with the positive control. The data suggest that such dose is as effective as the commercial dosages of diclofenac and paracetamol. The intraperitoneal injection of 1% acetic acid induced unpleasant stimuli observed from the writhing response of mice similar to rats subcutaneously injected with formalin. It is postulated that the release of PGE2 and PGF2α [8,9] along with TNF-α [10] may have been indirectly induced by irritant in peritoneal fluid and subplantar tissue of both positive control and *A. retusa* extract treated mice that may have possibly reduced COX enzyme activity [11] similar to the action of common non-selective COX inhibitors which is believed to be acting on lipooxygenase and/or cyclooxygenase in peripheral tissues [12]. Acute inflammation triggered by the specific action of λ-carrageenan promotes the accumulation pro-inflammatory factors such as prostaglandin which are important mediators of inflammatory reactions [13,14]. The synthesis of inflammatory mediators may have been possibly inhibited by limiting the production of necessary mediators to produce specific inflammatory responses [15]. The possible reduction of inflammatory mediator synthesis may be responsible for the non-stimulation of nociceptors. It is hypothesized that the possible inhibition of COX is involved in mediating peripheral neurologic aspects [12] of pain demonstrated in the visceral model and both peripheral and central neurologic desensitation [16] exhibited in the somatic model and inflammation may have been the possible mechanism involved. Further test, however and should be conducted to confirm such mechanism of action.

Rutaecarpine, a non-polar indolopyridoquinazolinone alkaloid isolated from *Evodia rutaecarpa* (Rutaceae) was documented to have anti-inflammatory and analgesic properties [11] which has been tied to its ability to inhibit COX-2 and prostaglandin synthesis [17]. It is most likely that *A. retusa* may also contain rutaecarpine, but further studies on isolation and purification of the extract is recommended since the major sources of this compound are members of the Rutaceae family such as *Hortia, Zanthoxylum, Phellodendron, Tetradium, Spiranthera, Vepris, Metrodorea, Bouchardatia,* and *Fagara*. [11,18,19,20,21,22].

4.0 CONCLUSION

The current study presents the analgesic and anti-inflammatory property of *Atalantia retusa* non-polar extract in mice and rats, respectively. In the somatic pain model, the median dose of the extract has demonstrated significant analgesic property acting centrally and peripherally which has surpassed the anti-nociceptive effect of Paracetamol (10mg/kg BW) and equaled the anti-inflammatory effect diclofenac (1.43 mg/Kg BW). Potency of the extract, however, seems to diminish at the highest dose tested, which is concomitant with the effects observed in the visceral model demonstrating the maximum dose effect. The findings indicate the dose 1.437 mg/Kg BW to be the most effective dose comparable to the positive control acting peripherally similar to the rat inflammatory model. The high impact bioactivity of *A. retusa* non-polar extract has a very promising potential as an analgesic and anti-inflammatory drug.

REFERENCES

[1] Eustice C., Eustice R. 2008. *The Facts of Analgesics (Painkillers) Analgesics: Drugs designed to relieve pain*. (website) http://arthritis.about.com/od/analgesic/a/factsanalgesics.htm

[2] Pertusiy R M. 2004. Selective Cyclooxygenase Inhibition in Pain Management, *Journal of American Osteopathic Association* **104**:19–24

[3] The Committee on Code of Practice of the Philippine Association for Laboratory Animal Sciences. 2002. *Code of Practice for the Care and Use of Laboratory Animals in the Philippines*.

[4] Abbot F. V., Franklin K. B. J., Westbrook R. F. 1995. The formalin test: scoring properties of the first and second phases of the pain response in rats. *Pain* **60**:91–102

[5] Inoue K., 1991. *Arzneim-Forsh/Drug Res*. **41**(1):235

[6] Ramachandran S, Anbu J, Saravanan M, Gnanasam SK, Sridhar SK. 2002. *Indian J Pharmacol Sci*; **64**:67.

[7] Mahmoudi M, Moetesza-Semnani K and Mojra E. 2008. Anti-inflammatory and antinociceptive activity of Thymus pubescens extract. *Fitoterapia*: **79**, 361–365.

[8] Deraedt R, Jouquey S, Delevalée F, Plahaut M. *Eur J Pharmacol* 1980;**61**:17.

[9] Deraedt R, Jouquey S, Benzoni J, Peterfalvi M. *Arch Int Pharmacodyn* 1976;**224**:30.

[10] Ribeiro RA, Vale ML, Thomazzi SM, Pachoalato AB, Poole S, Ferreira SH, et al. 2000. *Eur J Pharmacol*; 387:111.

[11] Lee S.H., Son J.K., Jeong B.S., Jeong T. C., Chang H.W., Lee E. S., Jahng, Y. 2008. Progress in the Study of Rutaecarpine. *Molecules* **31**:272–300

[12] Abbott FV, Melzack R. 1982. *Brain Res*;251

[13] Vinegar R, Scheirber W, Hugo R. 1969. *J Pharmacol Exp Ther*;**150**:328.

[14] Damas J, Remacle-Volon G, Deflandre E. 1986. *Arch Pharm*;**332**:196.

[15] Vane J, Botting R. 1987. Inflammation and mechanism of action of anti-inflammatory drugs. *FASEB J*. **1**, 89–96.

[16] Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. 1992. *Pain*;**51**:5.

[17] Moon T. C., Murakami M., Kudo I., Son K. H., Kim H. P., Chang H. W. 1999. A new class of COX-2 inhibitor, rutaecarpine from *Evodia rutaecarpa*. *Inflammation Research*. **48**:621–625

[18] Champagne, D.E., Koul, O., Isman, M.B., Scudder, G.G.E., Towers, G.H.N. 1992. Biological activities of Limonoids from Rutales. *Phytochemistry* **31** (2):377–394.

[19] Lima L. M., Perazzo F. F., Tavares Carvalho J. C., Bastos J. K. 2007. Anti-inflammatory and analgesic activities of the ethanolic extracts from *Zanthoxylum riedelianum* (Rutaceae) leaves and stem bark. *Journal of Pharmacy and Pharmacology* **59**:1151–1158

[20] Rashid B. A., Mustafa M. R., Hashim S., Zakaria M. B. 1995. Muscle relaxant effects of *Atalantia roxburghiana* on intestinal smooth muscle, *International Journal of Pharmacognosy* **33**:159–163

[21] San Miguel, E. 2003. Rue (Ruta L., Rutaceae) in traditional Spain: Frequency and distribution of its medicinal and symbolic applications. *Economic Botany*. **57**:231–244

[22] Wu T., and Chen C. 2000 Acridone Alkaloids from the root bark of *Severina retusa* in Hainan. *Chem. Pharm. Bulletin* **48**(1): 85–90

Evaluation of Antimicrobial activity of stem bark of *Ficus bengalensis* Linn. Collected from different geographical regions

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Abstract

The stem bark of *Ficus bengalensis* is reported to have antimicrobial activity, we planned our work as to evaluate and compare antimicrobial activity of alcoholic extracts of stem bark of *F. bengalensis* by cup and plate method collected from three different geographical regions viz. Delhi, Gujarat and Uttaranchal. The activity of all the extracts at the dose level 1000 µg/ml, 500 µg/ml and 100 µg/ml were found to be quiet different and all the extracts showed that activity is varied as geography and environmental conditions are changed.

Keywords: *Ficus bengalensis*, stem bark, antimicrobial activity.
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INTRODUCTION

Ficus bengalensis is an indigenous plant belonging to family Moraceae possessing varied pharmacological properties like antidiabetic, antimicrobial, antioxidant, antiseptic, gonorrhoea and also tender ends of hanging roots are prescribed to stop vomiting. The quality of medicinal plant depends on the geographical origin, time and stage of growth when collection has been done and post harvest handling. In this direction we collected the stem bark of *F. bengalensis* and planned our work to evaluate and compare antimicrobial activity of *Ficus bengalensis* Linn. by cup and plate |method using Ofloxacin as standard for microbial assay.

MATERIAL AND METHODS

Plant material

The stem barks of *F. bengalensis* Linn. Were collected from New Delhi (FB/DL), Gujarat (FB/GJ) and Uttaranchal (FB/UA) in the month of May 2008. The age of plant was found to be in the range of 25-30 years as enquired from local person. The specimen of collected bark was given for authantification in Raw Material and Laboratory of National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (voucher no. NISCAIR/Consult/RHMD/2008-09/1010/41).

The stem barks of FB/DL, FB/GJ and FB/UA were washed and dried in an electric oven at a temperature 40°C for 48 hours.

Preparation of the extracts

Dried barks are coarsely powdered and defatted with petroleum ether by soxhlet apparatus. Defatted drug than exhaustively extracted with 95% ethanol in soxhlet apparatus. The extract was concentrated under reduced pressure to get dark brown mass. The viscous dark brown mass is than dried in air as dried powered extract. (Sgrawat, H.et al. 2007, Edwin E. 2008). The percentage yield of ethanol extracts of stem barks of FB/DL, FB/GJ and FB/UA was found to be 6.6 %, 9.56 % and 7.0 % respectively. The Phytochemical screening of all the extracts was carried out for the presence of Alkaloids, Proteins & Amino acids, Carbohydrates, Flavonoids, Phenolic group, Glycosides, Saponins, Tannins, Steroids, Triterpinoids. (Cromwell B.T. et al. 1955, Kokate, C.K. et al. 1996, Finar I. L. 1975, Peach K. and Tracey M.V.1955, Geinssman T.A. et al. 1955, Trease, G.E. and Evans, W.C 1989) It was found that proteins and amino acids, carbohydrates, flavonoids, Phenolic groups, glycosides, saponins, tannins, steroids and triterpenoids were present and alkaloids were absent in all the extracts.

Test organism and inoculums

Escherichia coli (NCTC-6571) and Staphylococcus aureus (NCTC-10418) were obtained from the Department of Pharmaceutics (Microbiology), B.M.Shah college of Pharmaceutical Education and Research, Modasa. Ofloxacin was taken as standard 25 mg/ml (Gupta K.C., Viswanathan R., 1956) obtained from the Megh Pharmaceuticals, Modasa.

Dehydrated nutrient agar media was prepared in distilled deionized water. Test organisms were prepared and cup and plate method was used for microbial assay. (Indian Pharmacopoeia 1996). Three doses of all the drugs (1000 µg/ml, 500 µg/ml and 100 µg/ml) were taken and effects were compared against the zone of inhibition.

RESULT AND DISCUSSION

All the extracts showed antimicrobial activity against *E. coli* and *S. Aureus* though the geography is different but FB/UA shows more antibacterial activity against *E. coli* at the concentration of 500 µg/ml and 1000 µg/ml while FB/GJ shows more antibacterial activity against *S. aureus* at the concentration of 500 µg/ml and 1000 µg/ml. Antibacterial activity were not shown at the concentration

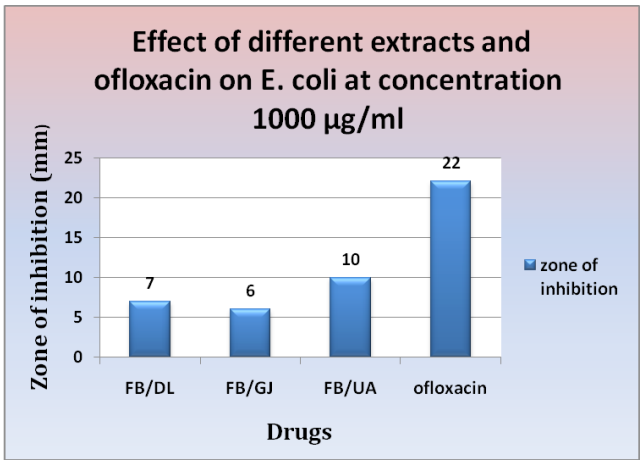

Figure 1: Antimicrobial activity against *E.coli* at 1000 µg/ml conc..

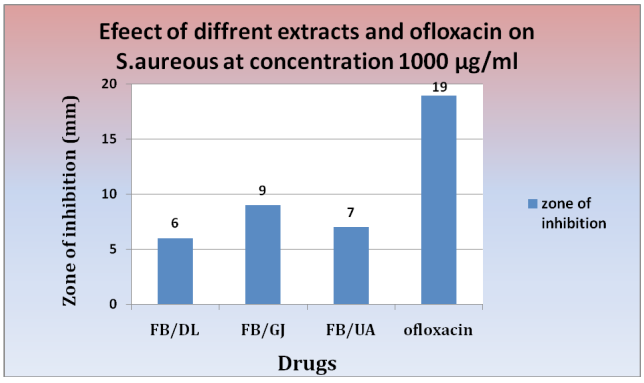

Figure 2: Antimicrobial activity against *S.aureus* at 1000 µg/ml conc..

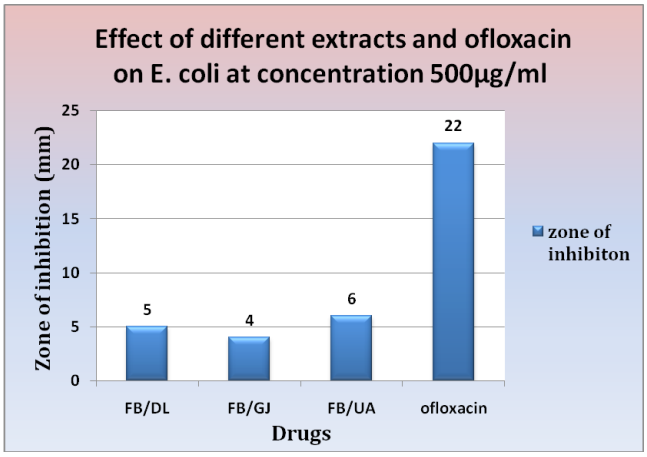

Figure 3: Antimicrobial activity against *E.coli* at 500 µg/ml conc..

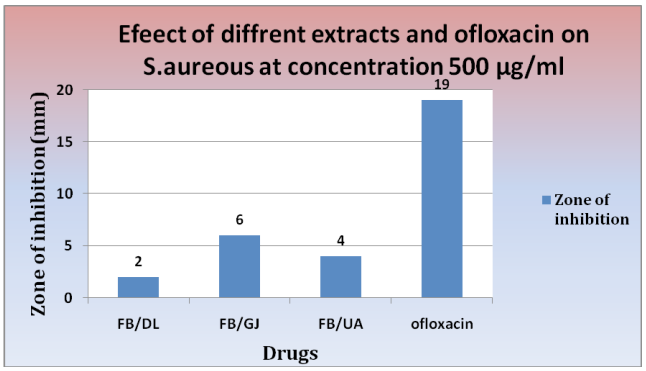

Figure 4: Antimicrobial activity against *S.aureus* at 500 µg/ml conc..

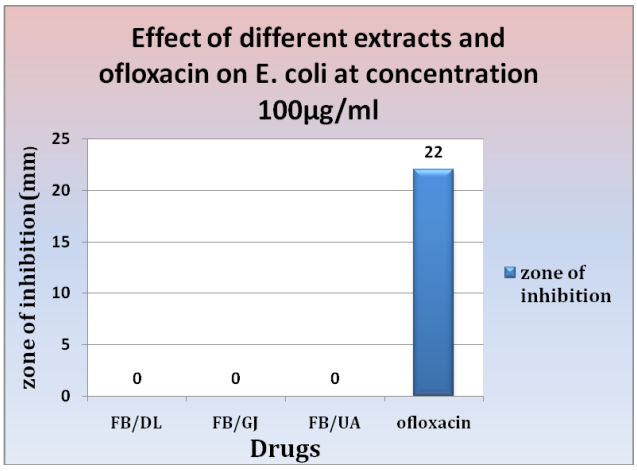

Figure 5: Antimicrobial activity against *E.coli* at 100 µg/ml conc..

Table 1.1: Antimicrobial activity of stem barks of *Ficus bengalensis*

Drug	Sample Code	Sample Conc. (µg/ml)	Zone of Inhibition (mm) E.coli	Zone of Inhibition (mm) S.aureus
FB/DL	A	1000	7	6
	B	500	5	2
	C	100	0	0
FB/GJ	A	1000	6	9
	B	500	4	6
	C	100	0	0
FB/UA	A	1000	10	7
	B	500	6	4
	C	100	0	0
Ofloxacin	Std	Standard	22	19
Control	Co.	-	-	-

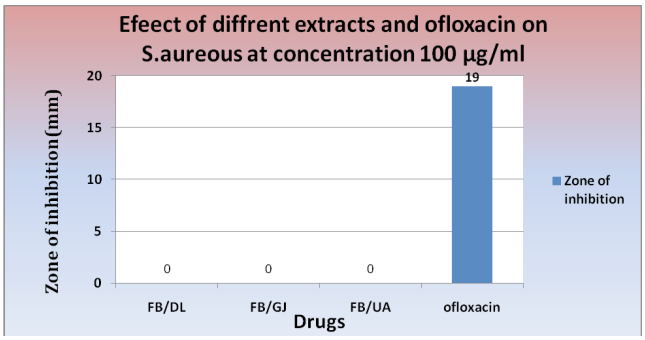

Figure 6: Antimicrobial activity against *S.aureus* at 100 µg/ml conc..

of 100 µg/ml by all extracts. Results are given in table 1.1 and figures 1-6.

REFERENCES:

1. Cromwell B.T., Peach K., Tracey M.V., 1955. Modern Methods of Plant Analysis. In: *alkaloids*.; Vol.**3**, 1 eds, Berlin, Springer Verlag, pp. 373–374.
2. Edwin E., Sheeja E., Chaturvedi M., Sharma S., Gupta V.B., 2008. A Comparative study on anti hyperglycaemic activity of Ficus bengalensis, Linn aerial roots and barks. *Phcog Mag*, **4**(13), pp. 95–97.
3. Finar I. L., 1975. *Organic chemistry Stereochemistry and Chemistry of Natural Products*. Volume **II**. Longman Scientific and Technical press, London, p. 518.
4. Geinssman T.A., 1955. Flavonoids. In: Peach, K., Tracey M.V., editors. *Modern Methods of Plant Analysis* Vol. **3**, Berlin; Springer Verlag: pp. 34–36.
5. Gupta K.C., Viswanathan R., 1956. Antituberculous substances from plants. *Antibiot. & Chemother.*, **6**, pp. 194–195.
6. *Indian Pharmacopoeia* (1996). Controller of Publication, Delhi, Vol. **II**, A-101.
7. Kokate, C.K., Purohit A.P. Gokhale S.B., 1996. Pharmacognosy. In:*alkaloid*.30 eds. Nirali Prakashan, Pune, p. 447.
8. Peach K., Tracey M.V., 1955. *Modern Methods of Plant Analysis* Vol. **4**. Berlin: Springer Verlag; p. 68.
9. Sgrawat, H., Sharma S., Chaturvedi M., Edein E., Shukla S., 2007. Evaluation of the phytochemicals and antidiabetic activity of Ficus bengalensis. *int J Diab Dev Ctries*, **27**(2), pp. 56–59.
10. Trease, G.E., and Evans, W.C., 1989. Pharmacognosy. In: *Phenols and Phenolic Glycosides*, ELBS, London, pp. 223–224, 246–249.

Figure 6: Antimicrobial activity against *S.aureus* at 100 µg/ml conc..

Antibacterial activities of genetic variants of *Mirabilis jalapa*

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Abstract

The aqueous and ethanolic extract derived from the leaves of an indigenous medicinal plant *Mirabilis jalapa L* (white, cream, yellow and pink, flowered plants) were screened for antibacterial activities against *Staphylococcus aureus*. Aqueous extracts did not display any inhibition to the tested bacteria. However, the ethanolic extract of only white flowered plant showed good antibacterial activitiy against *S. aureus* i.e. 54%. While other three colors i.e. cream, yellow, and pink flowered plants extract didn't show any zone of inhibition against the tested bacteria. The growth inhibitions (%) were calculated with reference to the activities of tetracycline which was taken as a standard (100%). The research clear cut indicates the effectiveness of white flowered plant of *M. jalapa* against *S. aureus*. Which is responsible for causing diseases like skin infections, pneumonia, and food poisoning etc.

Keywords: *Mirabilis jalapa*, antibacterial activitiy, white, cream, yellow and pink flowered plants.
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INTRODUCTION

The plant *Mirabilis jalapa* (Nyctaginaceae) is a tall herbaceous climbing plant with opposite leaves, large showy flowers, coriaceous obovoid fruits and prominent tuberous roots, planted as an ornamental plant throughout the country [1].A special quality of this plant is that, flowers of different colors can be found simultaneously on the same plant, i-e several colors of flower are available i.e. white, yellow, cream and pink, etc or an individual flower can be splashed with different colors. Another interesting thing is a color-changing phenomenon. For example, in the yellow flower, as the plant matures, it changes to a pink color. Similarly white flowers can change to light violet, which shows phenotype change.

M. jalapa has been used in traditional medicine. It is extensively used for treatment of dysentery, diarrhea, conjunctivitis, edema, inflammation, swellings, muscular pain, swelling and abdominal colic, also used as a laxative by people from different countries [2-6].The extract of *M. jalapa* have also been reported to possess various bioactivities including antibacterial, antiviral, antifungal, protein synthesis inhibition, antimicrobial, antinociceptive, and antigonorrhoeal antispasmodic, diuretic, carminative, cathartic, hydragogues, purgative, stomachic, tonic and vermifuge properties [2, 7–15].

Several constituents have been isolated from the root and aerial parts of this plant including some rotenoids, an isoquinoline derivate, terpenoids, steroids, phenolic compounds, d-glucoside, ursolic acid, mirabalisoic acid, trigonellin, an antiviral protein, alanine, alphaamyrins, arabinose, beta amyrins, campesterol, daucosterol and dopamine [7, 16–23].

The medicinal properties of *M. jalapa* have been well characterized in several studies. A protein purified from the root tubers of *M. jalapa* was confirmed to be an antiviral protein. This has been shown to inhibit the mechanical transmission of tomato mosaic virus (TMV) in tobacco, tomato, and pepper plants, and cucumber green mottle mosaic virus in cucumber plants [24–29]. While the protein has also an inhibitory effect on cell-free protein synthesis and an antiproliferative effect on tumor cells, it inhibits in vitro protein synthesis of prokaryotes and eukaryotes [30–32].

In the current study, we present the results of antibacterial activitiy of *M. jalapa* according to the flower colors. This study was designed with a view to explore antibacterial action of *M. jalapa* due to flower

Table 1.

Extraction base	Bacteria	Inhibition % age			
		White color flower	Cream color flower	Yellow color flower	Pink color flower
Water	*S. aureus*	0	0	0	0
Ethanol	*S. aureus*	54	0	0	0

colors, and to find the most effective type of *M. jalapa* against *Staphylococcus aureus,* which is gram positive cocci responsible for causing diseases like skin infections, pneumonia, and food poisoning etc, in such a large Varity of flowers. Therefore, four different flower colors of *M. jalapa* were screened for antibacterial activities.

MATERIAL AND METHODS

Plant Material

The plant *M. jalapa* of various flower colors (fresh leaves) were collected from Abbottabad, Pakistan in the month of October. The specimens were identified through herbarium Hazara University Mansehra Pakistan.

Preparation of extracts

The shade-dried plant material was chopped into small pieces and then pulverized into fine powder (100gms each) and was soaked (each flower color plant) in water and ethanol separately for about 4 weeks. Both the extracts of each flower colors were separately filtered and evaporated under reduced pressure to yield a gum (8-13gms aqueous and 4-7gms ethanolic).

Antibacterial activities

The extracts were screened against human pathogen *S. aureus* by agar well diffusion method. Nutrient agar plates were swabbed with a 2-8 h broth culture of respective bacteria. Wells (6 mm diameter) were cut in the medium in each of these plates using a sterile metallic borer with centers at least 24 mm apart. Samples (100 mg/ml) and standard Tetracycline (100mcg/ml) were then added in their respective wells using sterilized dropping pipettes.
The antibacterial activity of each flower color of *M. jalapa* extract against the selected bacteria were compared to tetracycline, which was taken as a standard (100%).All the growth inhibitions (%) were calculated with reference to the activities of tetracycline by using the formula,

$$\%\text{Inhibition} = 100 \square \frac{zone\,of\,inhibition\,of\,the\,sample\,(mm)}{zone\,of\,inhibition\,of\,the\,Std\,(mm)} \times 100$$

RESULTS AND DISCUSSION

Antibacterial activities of *Mirabilis jalapa* plant (flower color variants) has been evaluated in vitro against *S. aureus*. The antibacterial activity of the extracts both water and ethanolic and their potency was assessed by the presence or absence of inhibition zone as displayed in Table 1. Aqueous extracts did not display any inhibition to the tested bacteria. However, the ethanolic extract of only white flowered plant showed good antibacterial activitiy against *S. aureus* i.e. 54%. While other three colors i.e. cream, yellow, and pink flowered plants extract didn't show any zone of inhibition against the tested bacteria. The growth inhibitions (%) were calculated with reference to the activities of tetracycline which was taken as a standard (100%).

From this research the effective type of plant in large variety of *M. jalapa* (flower color variants) against *S. aureus* has been identified. Only the white flower ethanolic extract gave best result by inhibiting the selected bacteria. Which is gram positive coccus responsible for causing diseases like skin infections, pneumonia, food poisoning etc. While the other three colors cream, yellow, and pink was not able to produce good result by inhibiting *S. aureus*. The research clear cut indicates that the *M. jalapa* is a beneficial medicinal plant have a powerful action against wide range of microorganisms as described [33]. The antimicrobial activity of *M. jalapa* was known from the vary past [2, 7, 8, 9, 10, 12] but a range of flower colors are available, in which four colors were checked for antibacterial screening. The result shows that each flower color plant have change in its action against microorganisms, i.e. only white color plant is effective against *S. aureus*.

The antimicrobial activities measured by M. Kola Oladunmoyo [33], as the ethanolic extract of the leaf of *M. jalapa* was tested for antimicrobial activity against five pathogenic bacterial strains: *E. coli, S. aureus, S. typhi, B. cereus* and *K. pneumoniae*. The highest zone of inhibition of leaf extract was 13.0 mm and the least 4.0 mm, but in the case of current study it has found that only white flower plant have inhibitory activity against *S. aureus*. However other colors didn't show any zone of inhibition against the

tested pathogen. The aqueous extracts were also not able to inhibit any of the tested bacteria. It may be because of the antimicrobial peptides, which may not be soluble in water. So by this way we can also say that *M. jalapa* is a broad spectrum antibiotic for certain microorganisms [8, 12]. The research is beneficial as it provides us the proper use of each plant (flower color) of *M. jalapa* in a large number of variants. From the above results it can be concluded that plant extracts have great potential as antibacterial compounds against microorganism and that they (specific color) can be used in the treatment of infectious diseases caused by these microorganism.

It was also reported that neither aqueous nor methanolic extracts of *M. jalapa* were able to inhibit any of the tested bacterial strains, i.e. *Ps. testosteroni, K. pneumoniae, M. flavus, P. morganii, B. subtilis and S. epidermidis* [34]. Same was the case in the current study for aqueous extracts which doesn't show any inhibitory activity, while the ethanolic extract of white flower was found to inhibit the tested bacteria.

It was also reported that M. jalapa have an antibacterial activities [33], but in the current study it was noted that only the White flower plant showed antibacterial activity against *S. aureus* and therefore only this plant can be used

Figure 1:

Figure 2:

Figure 3:

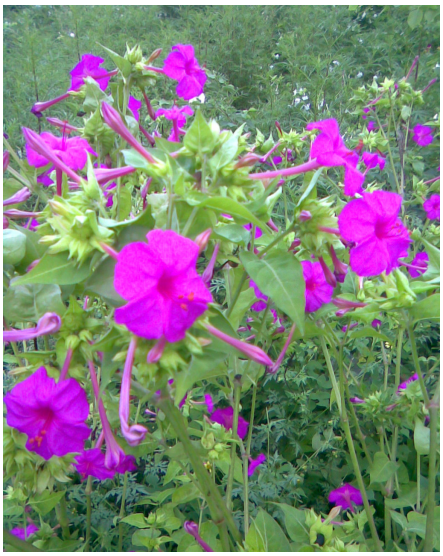

Figure 4:

White Flowered plant collected from shaikh-ul-bandi Abbottabad, Pakistan. While the other three colors Cream, Yellow and Pink flowered plants collected from Araam-Bagh Abbottabad Pakistan.

to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals against disease's caused by *S. aureus*. Other wise if we use Cream, Yellow or Pink against *S. aureus* it will be useless, have no activity. Such screening of various natural organic compounds and identifying active agents and the most effective form is the need of the hour. By the result of this research we can increase the efficacy of anti microbial agents obtained from *M. jalapa* by using the most effective type of selected flower color plant against required pathogen.

In this research as it has found the change of action on microorganism because of color variation. It is so beneficial experiment, because we have a lot of plants which have variation of flower colors. For example *Catharanthus rosea (Vinca)* etc. Then after this we can also apply the same experiment to other plants too. And can find (select) the most beneficial and effective variety in these plants.

With out the wasting of time and money in such a medicine which is useless or having less effectiveness, means appropriate use of drug would be prepared now onward from *Mirabilis jalapa,* if we use the most effective flower color plant whose effect we have required. And it would be given in Table 1.

REFERENCES:

[1] Ghani A. (2003) *Medicinal Plants of Bangladesh*, 2nd Ed. pp. 304 the Asiatic Society of Bangladesh, Dhaka.

[2] Encarnaci'on, D.R., Virgen, M., Ochoa, N., 1998. Antimicrobial activity of medicinal plants from Baja California *Sur. Pharmaceutical Biology* **36**, 33–43.

[3] M'arquez, A.C., Lara, O.F., Esquivel, R.B., Mata, E.R., Luna, A., 1999. *Plantas medicinales deM'exico II. Composici'on, usos y actividad biol'ogica*.UNAM, M'exico, pp. 968–969.

[4] Holdsworth, D.K., 1992. A preliminary study of medicinal plants of Easter Island, South Pacific. *International Journal of Pharmacognosy* **30**, 27–32.

[5] Comerford, S.C., 1996. Medicinal plants of two Mayan healers from San Andr'es, Pet'en, Guatemala. *Economic Botany* **50**, 327–336

[6] Moreira, F., 1996. *Plantas de curam: cudie da sua saude atraves de natura-za*, 5th edition. Hemus Editora LTDA, Sao Paulo, Brazil.

[7] Vivanco, J.M., Querci, M., Salazar, L.F., 1999. Antiviral and antiviroid activity of MAP-containing extract from *M. jalapa* roots. *Plant Disease* **83**, 1116–1121.

[8] Kusamba, C., Byamana, K., Mbuyi, W.M., 1991. Antibacterial activity of *M. jalapa* seed powder. *Journal of Ethnopharmacology* **35**, 197–199.

[9] De Bolle, M.F., Osborn, R.N., Goderis, I.J., Noe, L., Acland, D., Hart, C.A., Torrekens, S., Van Leuven, F., Broekaert,W.F., 1996. Antimicrobial peptides from *M. jalapa* and *Amaranthus caudatus*: expression, processing, localization and biological activity in transgenic tobacco. *Plant Molecular Biology* **31**, 993–1008.

[10] Oskay, M., Sari, D., 2007. Antimicrobial screening of some Turkish medicinal plants. *Pharmaceutical Biology* **45**, 176–181.

[11] Cort'es, A.A.R., Lara, Ch.B., Aoki, M.K., 2004. Screening and selection of plants by positive pharmacologic effect on jejunum muscular contractility. *Pharmaceutical Biology* **42**, 24–29.

[12] Dimayuga RE. Antimicrobial activity of medicinal plants from Baja California Sur/Mexico. *Pharmaceutical Biol* **36**: 33–43, 1998.

[13] Hamill FA, Apio S, Mubiru NK, Ziraba RB, Mosango M, Maganyi OW, Soejarto DD. (2003) Traditional herbal drugs of Southern Uganda, II: literature analysis and antimicrobial assays. *J Ethnopharmacol*. **84**(1): 57–78.

[14] Walker CIB, Trevisan G, Rossato MF, Franciscato C, Pereira ME, Ferreira J, Manfron MP. (2008) Antinociceptive activity of *M. jalapa* in mice. *J Ethnopharmacol*. **120**(2):169–175.

[15] Caceres A, Menendez H, Mendez E, Cohobon E, Samayao BE, Jauregui E, Peralta E, Carrillo G. (1995) Antiogonorrhoeal activity of plants used in guatemala for the treatment of sexually transmitted diseases. *J. Ethnopharmacol*. **48**(2): 85–88.

[16] Yi-Fen, W., Ji-Jun, C., Yan, Y., Yong-Tang, Z., Shao-Zong, T., Shi-De, L., 2002. New rotenoids from roots of *M. jalapa*. *Helvetica Chimica Acta* **85**, 2342–2348.

[17] Asprey, G.F., Thornton, P., 1955. Medicinal plants of Jamaica. Part II. *West Indian Medical Journal* **4**, 145–168.

[18] Stanic, G., Petricic, J., Todoric, A., Blazevic, N., 1988. Sterols in the root of *M. jalapa L. Acta Pharmaceutica Jugoslavica* **38**, 255–257.

[19] Siddiqui, S., Siddiqui, B.S., Adil, Q., Begum, S., 1990. Constituents of *M. jalapa*. *Fitoterapia* **61**, 471.

[20] Ali, M., Ansari, S.H., Porchezhian, E., 2001. Constituents of the flowers of *M. jalapa*. *Journal of Medicinal and Aromatic Plant Sciences* **23**, 662–665.

[21] Yang, S.W., Ubillas, R., McAlpine, J., Stafford, A., Ecker, D.M., Talbot, M.K., Rogers, B., 2001. Three new phenolic compounds from a manipulated plant cell culture, *M. jalapa*. *Journal of Natural Products* **64**, 313–317.

[22] Wei, Y., Yang, X.S., Hao, X.J., 2003. Studies on chemical constituents from the root of *M. jalapa*. *China Journal of Chinese Materia Medica* **28**, 1151–1152

[23] Yang SW. Three new phenolic compounds from a manipulated plant cell culture, *M. jalapa*. *J Nat Prod* **64**: 313–17, 2001.

[24] KATAOKA, J. *et al*. DNA sequences of Mirabilis antiviral protein (MAP), a ribosome-inactivating protein with an antiviral property, from *M. jalapa* L. and its expression in *Escherichia coli*. *J. Biol. Chem.*, v. **266**, p.8426–8430, 1991.

[25] KATAOKA, J. *et al*. Adenine depurination and inactivation of plant ribosomes by an antiviral protein of *M. jalapa* (MAP). *Plant Mol. Biol., Dordrecht*, v. **20**, p. 1111–1119, 1992.

[26] WONG, R. N. *et al*. Characterization of Mirabilis antiviral protein – a ribosome inactivating protein from *M. jalapa L. Biochem. Int.*, Marrickville, v. **28**, p. 585–593, 1992.

[27] MacBride, J. F. 1951. Euphorbiaceae, spurge family. Pages 543–544 in: *Flora of Peru*. Chicago Field Museum of Natural History, Chicago.

[28] Kubo, S., Ikeda, T., Imaizumi, S., Takanami, Y., and Mikami, Y. 1990. A potent plant virus inhibitor found in *M. jalapa L. Ann. Phytopathol. Soc. Jpn.* **56**:481–487.

[29] Takanami, Y., Kuwata, S., Ikeda, T., and Kubo, S. 1990. Purification and characterization of the anti-plant viral protein from *M. jalapa L. Ann. Phytopathol. Soc. Jpn.* **56**:488–494.

[30] MIYANO, M. *et al*. Crystallization and preliminary X-ray crystallographic analysis of *Mirabilis* antiviral protein. *J.Mol. Biol.*, London, v. **226**, p. 281–283, 1992.

[31] CAMMUE, B. P. *et al*. Isolation and characterization of a novel class of plant antimicrobial peptides from *M. jalapa* L. seeds. *J. Biol. Chem.*, Bethesda, v. **267**, p. 2228–2233, 1992.

[32] DE BOLLE, M. F. *et al*. Cloning and characterization of two cDNA clones encoding seed-specific antimicrobial peptides from *M. jalapa L. Plant Mol. Biol.*, Dordrecht, v. **28**, p. 213–721, 1995.

[33] Kola Oladunmoye M., 2007. Comparative Evaluation of Antimicrobial Activities of Leaf Extract of *Mirabilis jalapa*, *Trends in Medical Research* **2** (2): 108–112.

[34] Nair R, Kalariya T, and Chanda S, 2005 Antibacterial activity of Indian medicinal flora, Department of Biosciences, Saurashtra University, India, *Turk J Biol* **29**, 41–47.

Effect Of Hesperidin On Serum Glucose, HbA1c And Oxidative Stress In Myocardial Tissue In Experimentally Induced Myocardial Infarction In Diabetic Rats

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ABSTRACT

Present study was designed to evaluate in vitro antioxidant activity in heart and serum Glucose, HbA1c of Hesperidin on isoproterenol induced myocardial infarction in normal and diabetic in rats. Hesperidin (100 mg/kg, p.o) was administered for 28 days in rats injected with single dose of Streptozotocin (65 mg/kg, i.p, STZ) and nicotinamide (110 mg/kg, i.p, NIC) and after isoproterenol (200mg/kg, s.c., ISO) induced myocardial infarction in diabetic rats on 29th and 30th day. At the end of experimental period (i.e. on the day 31) heart tissue sample of each rat was collected and antioxidative parameter carried out for further estimations. Administration of STZ–NIC in rats showed a significant ($P<0.001$) increased in the levels of serum glucose, glycosylated heamoglobin (HbA1c). At the end of experimental period the level of malondialdehyde formation/ lipid peroxidation (LPO) and nitrite level in heart tissue was significantly increased. Whereas, the activity of biomarkers of oxidative stress such as reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were found to be decreased significantly compared to control rats. Treatment with Hesperidin significantly restored GSH level, SOD as well as catalase activity and reduced lipid peroxidation and nitrite in compared to diabetic control group. This study concluded that HES at 10 mg/kg may show reduced oxidative stress in heart on isoproterenol induced myocardial infarction in type 2 diabetic rats.

Keywords: Hesperidin, antioxidant, isoproterenol, Type 2 diabetic.

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INTRODUCTION

Three major metabolic abnormalities contribute to the development of hyperglycemia in Type 2 diabetes mellitus such as impaired insulin secretion in response to glucose, increased hepatic glucose production and decreased insulin-stimulated glucose uptake in peripheral tissues. The latter 2 abnormalities are primarily due to insulin resistance (1, 2). Type 2 Diabetes Mellitus is mainly characterized by the development of increased morbidity and mortality for cardiovascular disease. Cardiovascular disease is one of the leading causes of death in the western world and diabetes mellitus has been identified as a primary risk factor (3), due to which there is alteration in vascular responsiveness to several vasoconstrictors and vasodilators (4).

Oxidative stress has been associated with the pathogenesis of chronic diabetic complications including cardiomyopathy. The ability of antioxidants to inhibit these injuries has raised the possibility of newer therapeutic treatment for diabetic heart diseases.

Hesperidin (HES) is an abundant and inexpensive byproduct of Citrus cultivation and isolated from the ordinary orange Citrus aurantium and other species of the genus Citrus (family: Rutaceae). It is reported to have anti-allergic, radio protective, immunomodulator, anti-hypertensive and anti-oxidant properties. When hesperidin is administered orally, it is hydrolyzed by intestinal micro flora to yield a major active metabolite hesperidin.

So far in vitro antioxidant activity in heart of effect of Hesperidin on isoproterenol induced myocardial infarction in normal and diabetic in rats has not been

studied. Hence, the purpose of the present study was to instigate the effect of Hesperidin treatment on in vitro antioxidant heart tissue parameter alteration in Isoproterenol induced myocardial infarction in normal and type 2 diabetic rats.

MATERIAL AND METHODS

Drugs and Chemicals

Hesperidin was obtained from ACROS Lab, US. STZ and NIC were obtained from SIGMA, St. Louis, MO, USA. All other chemicals and reagents used in the study were of analytical grade.

Experimental Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Dharmaj Degree Pharmacy College, Anand. Sprague Dawley rats (210 ± 15 g) were housed in-group of 3 animals per cage and maintained under standardized laboratory conditions (12- h light/dark cycle, 24°C) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt., Pune) and purified drinking water *ad libitium*. The animal experiment was approved by Animal Ethical Committee of the Institute (1163/a/08/CPCSEA).

Experimental Induction of Type 2 Diabetes in Rats

Type 2 Diabetes was induced in rats by a single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg, STZ) in overnight fasting rats followed by the i.p administration of Nicotinamide (110 mg/kg, NIC) after 15 minutes. STZ was dissolved in citrate buffer (pH 4.5) and NIC was dissolved in normal saline. After 7 days following STZ and NIC administration, blood was collected from retro-orbital puncture and serum samples were analyzed for blood glucose (5). Animals showing fasting blood glucose higher than 300 mg/dL were considered as diabetic and used for the further study. Hesperidin (100 mg/kg, p.o) was administered for 28 days in diabetic rats and afterisoproterenol induced myocardial infarction in rats on 29th and 30th day.

At the end of experimental period (i.e. on the day 31) heart tissue sample of each rat was collected and carried out for further estimations.

Experimental Protocol

Animals were divided into following groups, each group containing 6 animals.

Group 1: Non-diabetic control [0.5 % Sodium CMC (1 ml/kg/day, p.o) as vehicle for 4 weeks and (ND-CON)] and normal saline subcutaneously on 29th and 30th day.

Group 2: Non-diabetic control treated with HES (100 mg/kg/day, p.o) as a suspension [0.5 % Sodium CMC for 4 weeks (ND-HES)] and normal saline subcutaneously on 29th and 30th day.

Group 3: STZ-NIC diabetic control [0.5 % Sodium CMC (1 ml/kg/day, p.o) as vehicle for 4 weeks (D-CON)] and received ISO (200 mg/kg, s.c.) on 29th and 30th day in normal saline.

Group 4: STZ-NIC diabetic rats treated with HES (100 mg/kg/day, p.o) as a suspension [0.5 % Sodium CMC for 4 weeks (D-HES)] and received ISO (200 mg/kg, s.c.) on 29th and 30th day in normal saline.

BIOCHEMICAL ESTIMATIONS

Characterization of Type 2 Diabetes Model

Type 2 diabetes was confirmed by measuring fasting serum glucose using standard diagnostic kit (SPAN diagnostics Pvt., India) and the degree of uncontrolled diabetic state was confirmed by measuring HbA1c (Ion Exchange Resin method). After 4 weeks, diabetes was confirmed by measuring glucose and HbA1c as mentioned above.

Estimation of biomarkers of Oxidative stress

The excised liver was then weighed and homogenized in chilled Tris buffer (10 mM, pH 7.4) at a concentration of 10% (w/v). The homogenates were centrifuged at 10,000 × g at 0 °C for 20 min using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the assay of following antioxidant parameters. The levels of Lipid peroxidation (LPO) formation and the activities of endogenous antioxidant enzymes such as catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) were estimated by the method of Slater and Sawyer (6) Hugo Aebi as given by Hugo (7) Moron et al (8) and Mishra and Fridovich (9). The clear supernatant was used for estimation of nitrite level (10).

Statistical Analysis

All of the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using a computer-based

fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

Characterization of Type 2 Diabetes

Single intraperitoneal (i.p) injection of Streptozotocin (65 mg/kg) followed by i.p administration of Nicotinamide (110 mg/kg) to rats produced severe hyperglycemia and increased HbA1c in 70 to 80 % the animals (Figure 1). The levels of glucose and HbA1c was significant (P<0.05) decreased after treatment with HES (100 mg/kg, p.o) alone and combination with HES (100 mg/kg, p.o) as compared to DB-CON rats.

Effect of HES on myocardial tissue parameter

There was a significantly (P<0.001) decrease in GSH level, (P<0.001) along with SOD and catalase activity (P<0.01) and increased lipid peroxidation (P<0.001) after myocardial infarction in STZ-NIC group (fig. 2). Treatment with Hesperidin significantly (P<0.05) restored GSH level, SOD as well as catalase activity and reduced lipid peroxidation significantly (P<0.05) in D-HES group compared to D-CON group (fig. 2). There was a significant ($P < 0.01$) increase in cardiac nitrite level in D-CON group as compared to ND-CON group after myocardial infarction. Hesperidin treatment in diabetic rats (D-HES)

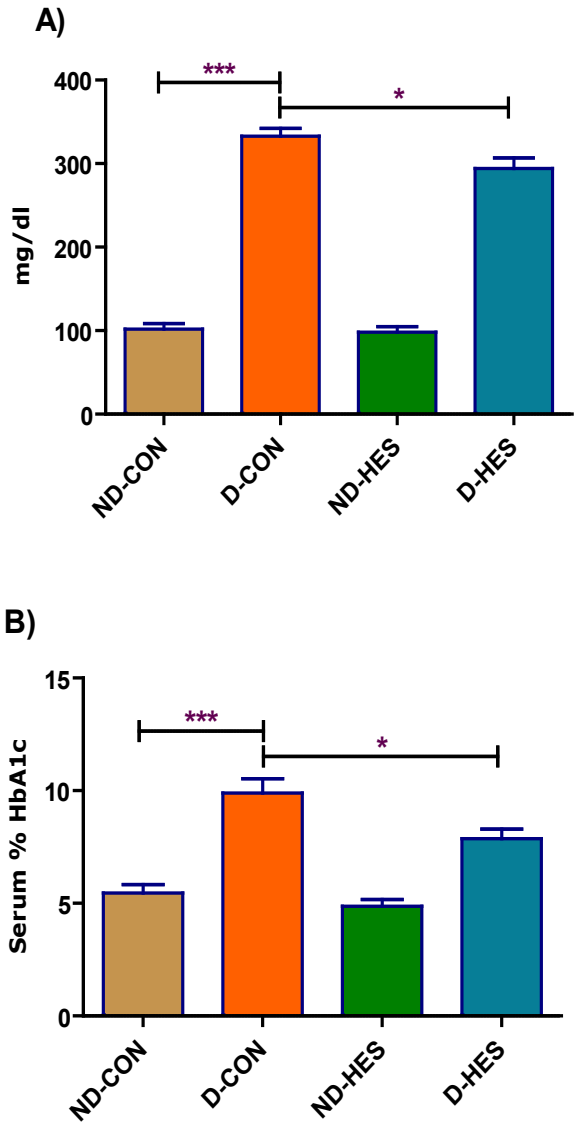
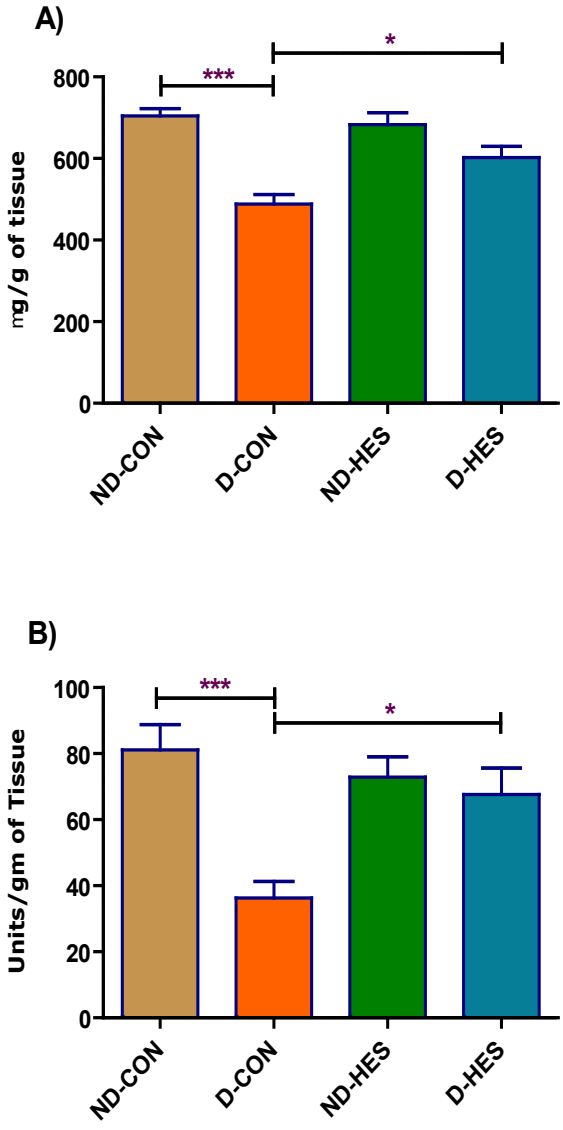
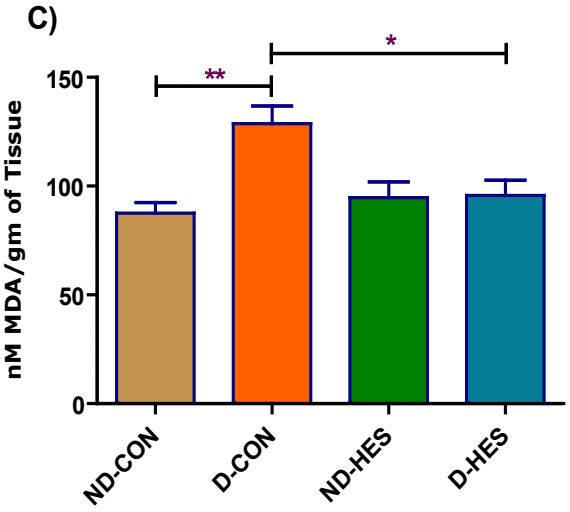


Figure 1: Effect of Hesperidin (100 mg/kg/day, p.o) on changes in serum glucose and HbA1c level in normal and STZ-NIC induced diabetic rats.
Values are expressed as mean ± SEM for six animals in the group. ***P<0.001, *P<0.05 considered statistically significant as compared to respective Control group.





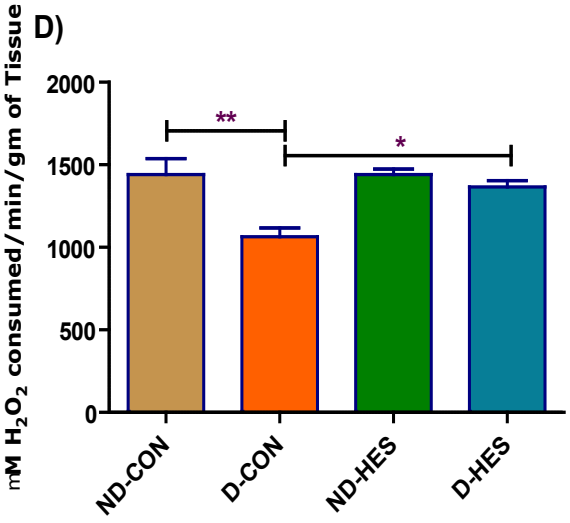


Figure 2: Effect of Hesperidin (100 mg/kg/day, p.o) on changes in GSH (A), SOD (B), MDA(C) and CAT (D) level after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.
Values are expressed as mean ± SEM for six animals in the group. *P<0.05, **P<0.01, ***P<0.001, considered statistically significant as compared to respective Control group.

significantly (P < 0.05) reduced nitrite level in heart as compared to D-CON group (Fig. 3).

DISCUSSION

The present study was under taken with the objective of exploring evaluate in vitro antioxidant activity in heart of Hesperidin on isoproterenol induced myocardial infarction in normal and diabetic in rats. Recent studies have suggested that prevalence of type 2 diabetes

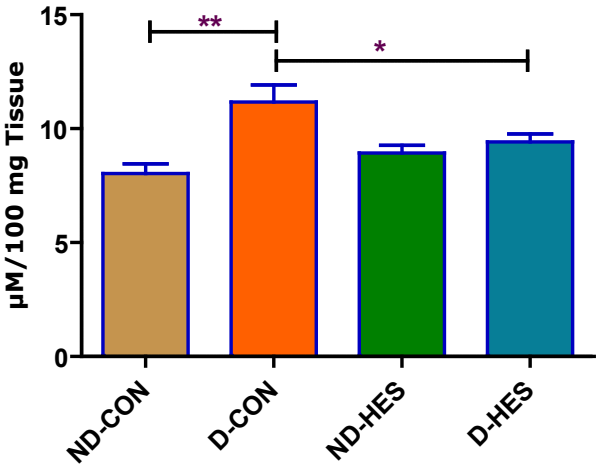


Figure 3: Effect of Hesperidin (100mg/kg/day, p.o) on myocardial changes in Nitrite level after completion of myocardial infarction in normal and STZ-NIC induced diabetic rats.
Values are expressed as mean ± SEM for six animals in the group. *P<0.05, **P<0.01, considered statistically significant as compared to respective Control group.

mellitus (T2DM) is rapidly increasing. T2DM is mainly characterized by the development of increased morbidity and mortality for cardiovascular disease (CVD) (11), so that it has been suggested that diabetes may be considered a cardiovascular disease (12). However, CVD risk is elevated long before the development of diabetes (13).

The release of ROS in the early phase of infarction, in combination with the ischemia induced decrease in anti-oxidant activity, renders the myocardium vulnerable. Previous studies proved that, ROS produced during myocardial infarction could trigger myocyte apoptosis by activating MAPK and produces DNA damage by activation of the nuclear enzyme poly (ADP ribose) polymerase, which consumes cellular Nicotinamide dinucleotide and adenosine triphosphate.

In the present study, an increase in the levels of serum glucose and HbA1c in STZ-NIC treated rats confirmed the induction of diabetes mellitus. Significant decrease was observed in the glucose and HbA1c level in diabetic rats after treatment with HES (100 mg/kg) when compared with diabetic rats (D-CON) at the end of experimental period.

Moreover, the levels of endogenous antioxidant (SOD, CAT and GSH) were reduced and lipid peroxidation increased in D-CON group showing increased oxidative stress. Similar results showing increased oxidative stress (increased lipid peroxidation and reduced SOD, CAT and GSH) have been reported in previous studies in STZ induced diabetes modal (14). The antioxidant

enzymes were restored significantly by HES treatment in STZ diabetic rats. HES at 100mg/kg may show improve antioxidative stress in heart experimentally induced myocardial infarction in type 2 diabetic rats.

Myocardial infarction causes further increase in oxidative stress and reduction in nitric oxide availability due to endothelial dysfunction. The destruction of nitric oxide is much prominent in STZ-NIC diabetic rats which is due to increase in ROS formation. Nitric oxide is rapidly inactivated by O_2- and it has been reported that an enhanced formation of O_2- radical may be involved in the accelerated break down of nitric oxide (15, 16). HES treatment reduces the nitrosative stress which is evident from myocardial nitrite level reduction. Thus it may be one of the reasons for the cardioprotective effect of HES against myocardial infarction.

There may be several mechanisms for cardioprotection by Hesperidin against Myocardial infarction. It may be due to reduction in hyperglycemia induced deleterious effects, reduction in NO destruction, utilization of glucose and restoration of endogenous antioxidants in STZ-NIC diabetic rats.

Administration of STZ-NIC caused decrease in SOD, CAT, and GSH but increase in MDA. Treatment with Hesperidin (100mg/kg, p.o) could improve result them. This study concluded that HES at 100 mg/kg may show reduced oxidative stress in heart and improve glucose and HbA1c on isoproterenol induced myocardial infarction in type 2 diabetic rats.

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REFERENCES:

1. Kahn SE, Porte DJ. The pathophysiology of type II (noninsulin-dependent) diabetes mellitus: Implications for treatment. In: Rifkin H, Porte DJ, eds. Ellenberg and Rijkin's Diabetes Mellitus: Theory and Practice. New York: *Elsevier Science* 1990:436–456.
2. Leibowitz HE. Oral hypoglycemic agents. In: Rifkin H, Porte DJ, eds. *Ellenberg nd Rijkin's Diabetes Mellitus: Theory and Practice*. New York: Elsevier Science 1990:554–574.
3. Uemura S, Matsushita H, Li W, Glassford AJ, Asagami T, Lee KH, et al. Diabetes mellitus enhances vascular matrix metalloproteinase activity: role of oxidative stress. *Circ Res 2001*; **88**:1291–8.
4. Senses V, Ozyazgan S, Ince E, Tuncdemir M, Kaya F, Ozturk M, et al. Effect of 5-aminoimidazole-4-carboxamide riboside (AICA-r) on isolated thoracic aorta responses in streptozotocin-diabetic rats. *J Basic Clin Physiol Pharmacol* 2001; **12**:227–48.
5. Masiello, P., Broca, C., Gross, R., Roye, M., Manteghetti, M., Hillaire-Buys, D., Novelli, M., Ribes, G. Experimental NIDDM: development of a new model in adult rats administered Streptozotocin and Nicotinamide. *Diabetes* 1998:**47**, 224–229.
6. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride and other halogeno alkanes or peroxidative reactions in rat liver fractions in vitro. *Biochem J* 1971; **123**:805–14.
7. Hugo EB. Oxidoreductases acting on groups other than CHOH: catalase. In: Colowick SP, Kaplan NO, Packer L, editors. *Methods in Enzymology*, vol. **105**. London 7 Academic Press, 1984; 121–5.
8. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta* 1979; **582**:67–78.
9. Mishra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biochem* 1972; **247**:3170–5.
10. Guevara S M, Iwanejko J, Dembinaka-Kiec A, Pankiewicz J, Wanat A, Anna P, et al. Determination of nitrite/nitrate in human biological material by the simple Griess reaction. *Clin Chim Act* 1988; **274**:177–88.
11. Gang Jee Ko, Young Sun Kang, Sang Youb Han, Mi Hwa Lee, Hye Kyoung Song, Kum Hyun Han, Hyoung Kyu Kim, Jee Young Han and Dae Ryong Cha. HESHEStazone attenuates diabetic nephropathy through an anti-inflammatory mechanism in type 2 diabetic rats. *Nephrology Dialysis Transplantation* 2008 **23**(9):2750–2760.
12. Kannel WB, McGee DL. Diabetes and cardiovascular diseases. The Framingham Study. *JAMA*. 1979; **241**:2035–2038.
13. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA*. 2001; **285**:2486–2497.
14. Xu Y, Gen M, Lu L, Fox J, Weiss S O, Brown R D, Perlov D, Ahmad H, Zhu P, Greyson C, Long CS, and Schwartz G G: PPAR-gamma activation fails to provide myocardial protection in ischemia and reperfusion in pigs. *Am J Physiol heart circ Physiol* 2005; **288**:H1314–H1323.
15. Graylewski R J, Palmer R M, Moncada S. Superoxidte anion is involved in the breakdown of endothelium derived vascular relaxing factor. *Nature* 1986; **320**: 454–456.
16. Rubanyi G M, Vanhoutte P M. Superoxide anions and hyperoxia inactive endothelium derived relaxing factor. *Am J Physiol* 1986; **250**:H822–H827.