Potent bioactive compounds from the ocean: some interesting aspects and applications

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ABSTRACT

Over seventy percent of the earth's surface is covered by oceans, and it is an established fact now, that life originated in the oceans. Additionally, the oceans are also the source of matchless natural products that are mainly accumulated in living organisms. Several bioactive compounds of therapeutic interest have been isolated from marine invertebrates, and some of them have been reported to be of microbial origin. Numerous of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds, primarily for deadly diseases like cancer, acquired immuno-deficiency syndrome, etc., while others have also been shown to possess several valuable properties. The secondary metabolites of microorganisms, algae and invertebrates, possess lifesaving properties, and are deadly toxins as well, depending on the dosage. The useful applications of these compounds, as bio-markers is also being explored, to further enrich pharmacological information. Recent developments have opened colossal areas of research for the isolation of biologically active compounds from marine flora and fauna.

Key Words: Marine natural products, seas, wonder drugs, marine invertebrates, bioactive secondary metabolites, fatal toxicity, bio-marker compounds, oceanic biodiversity.

INTRODUCTION

It is an undisputable fact that the development of drugs has greatly improved the quality and duration of human life. Chemical compounds, such as morphine, quinine, penicillin, streptomycin, reserpine, curare alkaloids, and digitalis, etc., led to treatments and even complete cures for diseases that were earlier considered to be fatal. The process of drug discovery continues today at a pace greater than ever before, and although sophisticated new approaches are used, nature continues to provide the biochemical insight forming the foundation of many newly developed drugs. One example is the recently approved anticancer drug taxol, a compound extracted from the bark of the North American Yew tree. Taxol, perhaps by virtue of its unique mechanism of action, has shown excellent results in treating several forms of cancer that were previously difficult to treat. Disappointingly, many of the "wonder drugs" generated over the past several decades have become less useful due to the development of drug resistance. Many pathogenic bacteria, once susceptible to antibiotics, have developed sophisticated biochemical methods to escape the effects of these drugs. A strain of drug-resistant mycobacterium, the pathogen that causes tuberculosis, for example, is almost totally resistant to our arsenal of antibiotic drugs. Some infections are, today, produced by bacteria that are immune to all known antibiotics. Similarly, some forms of cancer have evolved multiple drug resistance, making virtually all drug treatments ineffective.

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Global marine drug discovery scenario

Drug discovery efforts today, include the inhabitants of the world's oceans as a new source of biodiversity and novel drugs. In contrast to the terrestrial environment, little ethnomedicinal information is available to guide current marine research. With the exemption possibly of southern China, few societies have used marine organisms as crude drugs. Thus studies now in progress have relied on ecological observations of chemical defense and survival to identify those organisms that might be expected to contain drug candidates. Pharmacological investigations of oceanic organisms are relatively new and have been based on the establishment of unprecedented "scientific bridges" between the marine and pharmaceutical sciences. In this day and age, roughly one-half of all cancer drug discovery focuses

on marine organisms, and forecasts for the future are brilliant, as well. In fact, some of the most important recent discoveries have been from the oceanic milieu. Marine drug discovery began in the late 1970s by early investigators demonstrating unequivocally, that marine plants and animals were genetically and biochemically unique. Over 18,000 structurally unique and often highly bioactive metabolites have now been isolated from marine plants and animals. After the uniqueness of marine metabolism became accepted, programs began to evolve that linked academic marine scientists with biomedical researchers in the pharmaceutical industries. Programs, which established the foundations of today's efforts, were created in the 1980s in the United States, in Japan, and in Australia. Today these programs are expanding on the basis of their continuing discoveries of novel new drug leads. Unlike the majority of terrestrial drug research, marine drug discovery programs have been applied to selected, difficult to treat diseases that have eluded cures for decades. Yet, progress has been observed in many of these difficult areas. New drug leads have been identified with potent immunosuppressant properties, with anti-inflammatory properties, and with significant anticancer potential[1-2]. Perhaps the first molecule discovered was the unique cyclic ester bryostatin 1 isolated and defined by researchers at Arizona State University. Bryostatin 1 occurs as a trace component of the common bryozoan *Bugula neritina,* which occurs worldwide often as a conspicuous component of the fouling communities on pier pilings. The molecule was the most selective antileukemia agent, and recognized as a very potent inhibitor of numerous leukemia cells in culture. As is regularly the case, only selected populations of this illustrious animal were found to contain bryostatin 1. Bryostatin 1 has already been acknowledged as a chemically and pharmacologically exceptional molecule of great interest in basic medical research[3]. This compound possesses unprecedented immunostimulatory properties, and it activates protein kinase C, an important regulator of hormone-mediated signal transduction, and a novel enzyme target for the development of new antitumor drugs[4]. In the 1970's, as part of an NCI-sponsored survey of Caribbean invertebrates, the impressive cytotoxic properties of extracts of the mangrove ascidian *Ecteinascidia turbinata* were discovered. Although it was clear, even then, that this animal contained substances of great importance, the difficulty encountered in isolating and identifying the active substance(s) rendered this project virtually unsolvable, due to the fact that active substances were present in vanishingly small amounts, and the compounds were apparently of a very new and difficult to isolate structural class. After 20 years of advancements in chemistry, the active substances, named the ecteinascidins, were isolated and described by researchers at the University of Illinois and the Harbor Branch Oceanographic Institution[5]. The most abundant compound, Ecteinascidin 743, showed excellent potency, IC_{50}, = 0.5 ng/ml against murine (L-1210) leukemia *in vitro* and significantly extended the life spans of mice infected with P-388 lymphocytic leukemia. In subsequent testing, this compound showed selectivity towards MXI human mammary tumors cultivated in mice. Unlike bryostatin 1, ecteinascidin 743 is chemically related to a rare group of microbial antibiotics, the saframycins, which has raised the question of a possible microbial source existing within the tissues of this ascidian[6]. Another Caribbean ascidian, *Tridemnum solidum,* has also been recognized to contain substances of potential use in cancer chemotherapy. This ascidian was found to contain a series of cyclic peptides, the Didemnins, all of which were closely related. The most medicinally important of these compounds, Didemnin B, showed impressive cytotoxicity against lymphomas and significantly extended the survival of mice in the P-388 leukemia assay. On the basis of these encouraging properties, a large-scale collection of this animal was undertaken and larger amounts of Didemnin B were isolated[7]. Unfortunately, Didemnin B has subsequently been found to exhibit significant toxicity at doses near those required for life conservation. It is important to point out, however, that the evaluation of new drugs is a complex process in which both negative and positive results are continuously evaluated over time. Taxol, for example, required over 20 years of study before it was approved as a cancer drug. This fact has been once again realized during the development of Didemnin B. A more recent addition to the list of thrilling marine anticancer agents is dolastatin 10, a linear peptide discovered by researchers at Arizona State University from the sea hare *Dolabella auricularia*, collected in the Indian Ocean. Found in complex mixtures with related peptides, dolastatin 10 showed outstanding inhibitory effects against several forms of skin cancers in laboratory, studies. More importantly, subsequent whole animal testing showed this peptide to provide significant effects in controlling human melanoma in implanted mice[8]. The element of curiosity in this work is the true origins of the peptides in *D. auricularia.* Sea hares are most often herbivores, and it has been unequivocally demonstrated that these shell-less mollusks acquire defensive chemicals from their diets rather than synthesizing them as many animals do. Thus Dolastatin 10 and its analogs are most likely of an algal dietary origin. Based on our knowledge of the chemistry of marine algae, the Dolastatins are likely to be produced by filamentous blue-green algae (cyanobacteria), which are often abundant in these habitats.

Many of these compounds have possessed important biomedical properties, but only recently was a sponge-derived compound, halichondrin B, added to the list of agents to enter clinical trials. Halichondrin B, a novel polyether, was isolated and first identified in Japan. This remarkable metabolite was discovered in the sponge *Halichondria okadai*, various collections of which have yielded a variety of diverse toxins. Halichondrin B is a novel

compound, related most closely to several of the toxins produced by toxigenic dinoflagellates[9]. Due to this relationship, and for the reason that *H. okadai* had been found to concentrate okadaic acid, a metabolite of the dinoflagellate *Prorocentrum lima*, it seems logical that the compound may also be of dinoflagellate origin probably acquired by the filter feeding process. Sustaining this concept, halichondrin B has recently been isolated by several investigators from a variety of sponges. Halichondrin B shows selective antitumor effects against human ovarian cancers in mice, as well as activity against melanoma and various forms of leukemia. It seems clear that Halichondrin B is one of the most promising new anticancer drugs isolated to date. Another examples of molecules being intensely studied are discodermolide, a unique immunosuppressive and cytotoxic agent, isolated by Harbor Branch Oceanographic Institution scientists from the deep water sponge *Discodermia dissolute*[10] and curacin, a novel anticancer agent from the Caribbean bluegreen alga *Lyngbya majuscula,* discovered by researchers at Oregon State University[11]. Discodermolide has been shown to possess the identical tubulin polymer stabilizing properties as taxol, which certainly indicates that comprehensive preclinical studies should be undertaken[12]. Among recent discoveries of significance, Sebastianines A and B, two novel pyridoacridine alkaloids with fresh ring systems, have been isolated from the tunicate *Cystodytes dellechiajai*[13], and a novel cytotoxic 16-membered macrodiolide, Amphidinolide X, has been isolated from a marine dinoflagellate *Amphidinium* sp[14].

Setbacks: the toxicity factor

As these compounds (mainly secondary metabolites) are cytotoxic, they inherently possess fatally toxic properties, on several instances. As a result, in due course, some of them were prematurely withdrawn from the clinical trials due to life-threatening toxic side effects in the patients. Didemnin B, as indicated earlier, has shown ineffectiveness to moderate anticancer response in different target sites and always invariably accompanied with high toxicity to the patients[15]. However, ultimately due to its extreme toxicity, it was withdrawn from the phase II clinical trials[16]. Moreover, Dehydrodidemnin B or aplidin, an oxidation product of didemnin B, isolated from Mediterranean tunicate *Aplidium albicans* with apparently more potent anticancer potential is also being developed for its phase I clinical trials in Europe[17].

Similarly, girolline and jaspamide, isolated from the sponge *Pseudoaxyssa cantharella*[18] and the Indo-Pacific sponge *Jaspis splendus*[19], respectively, were also withdrawn from the clinical trials due to their extremely toxic side effects. Girolline resulted in hypertension problems in the patients while jaspamide was withdrawn benevolently from the preclinical evaluation stage as it was excessively toxic[20]. Yondelis, a promising anticancer compound, currently in phase III clinical trials, has been approved as an Orphan Drug[21]. Conversely, significant hepato- and hemato-toxicities of Yondelis in rats, mice, and monkeys could in fact limit its prospective use as an anti cancer drug in humans[22].

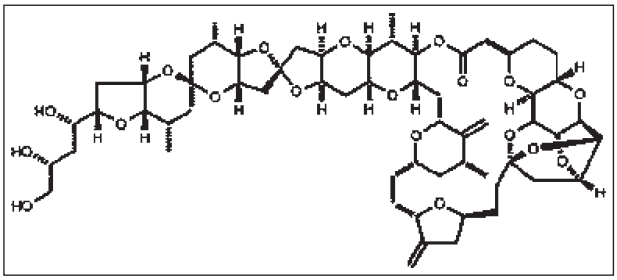
However, recent study showed that high dose of dexamethasone offered complete protection against the hepatotoxicity in rats by yondelis. Another anticancer compound, LU103793, a dolastatin 15 analogue, has failed to show activity in patients with melanoma and breast cancer in phase II trials, however, trials are continuing in ovarian, prostate, and colon cancer patients[23]. Besides their therapeutic efficacy studies in tumor xenograft models, these compounds along with many others are yet to be evaluated for their anticancer activities in the chemical/oncogene-induced animal carcinogenesis models. Additionally, a useful application of marine chemical compounds, emerging from their potential to induce/modulate adduct formation, with susceptible biological entities, like the DNA, certain enzyme systems, proteins, etc., can be beneficially employed to study the mechanism of carcinogenesis, as well as the anticancer mechanism of action of mainly newer drugs. Sarcophine, a furanocembrane diterpene, isolated from the Red Sea soft coral *Sarcophyton glaucum*, was found to act as an effective inhibitor of JB6 cell transformation, presented an example of adduct formation, possibly being induced by the compound itself[24]. Several such compounds can be used suitably as bio-markers to study the adduct formation, facilitating a better understanding of the mechanism carcinogenesis, and drug action, necessary for the development of better drugs.

CONCLUSIONS

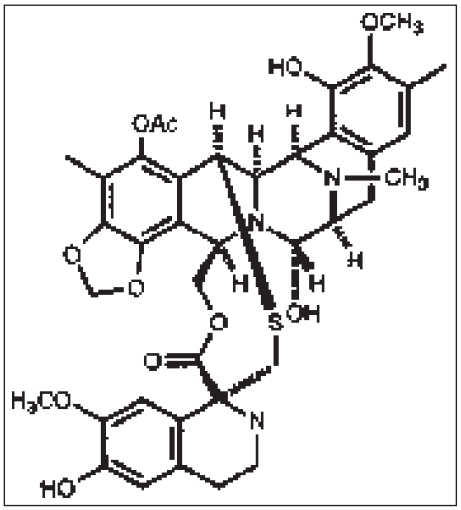
In discussions of drugs of marine origin, it is important to distinguish between molecules providing "drug leads" and those molecules more adequately described as "drug candidates" that are presented here. Despite of the fact that several pharmacologically active marine compounds have been dropped from further drug developments, on account of severs toxicities, still there is a vast scope of finding new drug leads from this colossal source. Marine plants and animals have provided literally hundreds of compounds that can be defined as the former, but few have advanced to the stages of clinical trials. The molecules presented here are thus concrete examples of the exciting advances being made in marine drug discovery. The process discovery and identification of these compounds suggests that they have already contributed significantly to biomedical research and at least some of them may indeed reach the status of clinical trials. Marine drug discovery can prove to be an immensely useful ray of hope for the most dreaded

diseases, including cancer. The discovery and invention of a new class of anti cancer agents, known as the vascular disrupting agents, such as the Combretastatins and Sorafenib, also give a ray of hope to cancer patients[25]. With the marine drug research, making speedy advances, we can expect similar agents from the ocean anytime now. Moreover, the search for better anticancer agents must go on at a war footing even in the lesser investigated marine environments, like the Indian Ocean, where huge unexplored opportunities still lie unearthed[26]. A thoroughgoing war against cancer, at all fronts is the only viable option left with us.

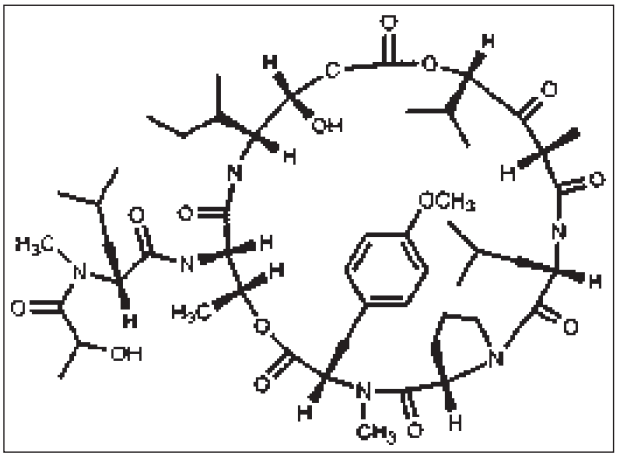
What appears likely is that studies of new drug leads from marine sources will significantly expand. As the scientific bridges between marine science and drug discovery continue to be built, new collaborations will lead to expanded pharmacological research. There is no doubt that these global studies will identify novel marine drug candidates in diverse areas of therapeutic development. When one considers the extent of oceanic biodiversity, including such major, uninvestigated groups as marine bacteria and fungi, it seems likely that marine sources could be the major source of new drugs for the succeeding years.



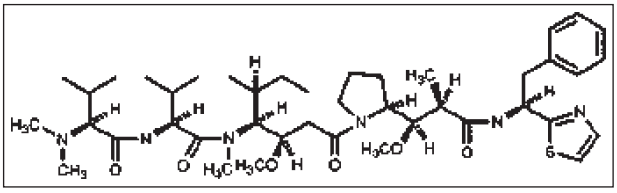
Halichondrin B



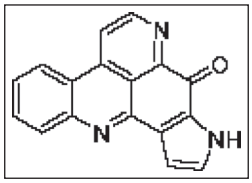
Ecteinascidin 743 (Yondelis)



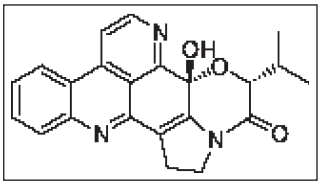
Didemnin B



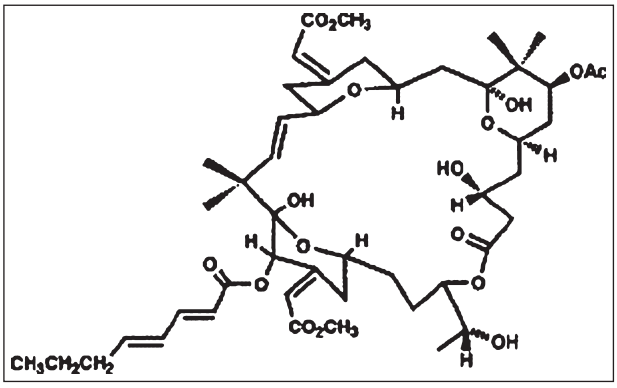
Dolastatin 10



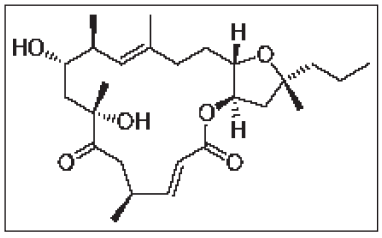
Sebastianine A



Sebastianine B



Bryostatin 1



Amphidinolide X

Some potent bioactive compounds from the ocean

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Endangered Medicinal Plant *Embelia ribes* Burm.f.- A Review

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ABSTRACT

Embelia ribes Burm. f. belongs to the family Myrsinaceae found in hilly parts of India up to 1500 m. elevation from outer Himalayas to Western Ghats. It is an endangered medicinal plant valued for its digestive, carminative, anthelmintic and laxative property since time immemorial. It is also used in diabetes, heart related problems, nerval disorders, cancerous tumors and liver disorders. The seeds are also used for wound healing antioxidant, anti-inflammatory, analgesic and contraceptive activity. Due to over exploitation of this plant it is reported in red list data book as vulnerable [Ravikumar & Ved 2000]. Therefore an overview of this plant on pharmacognosy, pharmacology, safety and toxicity is presented below along with HPLC details of Embelin the active constituent of the seeds.

Key words: *Embelia ribes,* Myrsinaceae, Pharmacognosy, Pharmacology

INTRODUCTION

Embelia ribes Burm. (Myrsinaceae) is a large woody tropical forest scandent shrub with slender branches and gland-dotted leaves[1]. It is distributed in moist deciduous forests of the Western Ghats of South India, Jammu & Kashmir, Himachal Pradesh, Uttar Pradesh, Assam and Maharashtra, Sri Lanka, Malaya, Singapore and South China[2,3,4]. In historical purview it is narrated in ancient classical texts of Charaka, Sushruta, and Vagbhatta where it is recommended mainly as a krimighna. It is also considered digestive, carminative, laxative and useful in dropsy and pneumonia[4]. It is a popular adjuvant in most of the herbal formulas. The plant contains quinone derivative embelin (3-Undecyl 2, 5-dihydroxy, 1, 4-benzoquinone), quercitol, and fatty ingredients; an alkaloid, christembine, a resinoid, tannins and minute quantities of a volatile oil[2,4,5]. The dried fruits are being used for preparation of medicine. It is widely used as anti-helminthic, anti-carminative, anti-bacterial, anti-inflammatory, anti-diuretic and anti-astringent[1,6]. Embelin has been also reported as a potent inhibitor of NF-κB activation, which makes it a potentially effective suppressor of tumor cell survival, proliferation, invasion, angiogenesis, and inflammation and has great potential as a therapeutic agent for osteoporosis and cancer-linked bone loss[7,8]. Recent findings also suggest embelin as a novel adjuvant therapeutic candidate for the treatment of hormone-refractory prostate cancer that is resistant to radiation therapy.[9]

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Morphology

Embelia ribes Burm. f., is a large scandent shrub; branches long, slender, flexible, terete with long internodes, the bark studded with lenticels. Leaves coriaceous, 5-9 by 2-3.8 cm., elliptic or elliptic lanceolate, shortly and obtusely acuminate, entire, glabrous on both sides, shining above, paler and somewhat silvery beneath, the whole surface covered with scattered minute reddish sunken gland (conspicuous in the young leaves), base rounded or acute; main nerves numerous, slender; petioles 6-16 mm. long, more or less margined, glabrous. Flowers penta-merous, numerous, small, in lax panicled racemes which are terminal and from the upper axils; branches of the panicle often 7.5-10 cm long with more or less glandular pubescent; bracts minute, setaceous, deciduous. Calyx is about 1.25 mm. long; sepals connate about 1/3rd of the way up, the teeth 5, broadly triangular-ovate, ciliate. Petals 5, greenish yellow, free, 4 mm. long, elliptic, subobtuse, and pubescent on both sides. Stamens 5, shorter than the petals, erect; filaments inserted a little below the middle of the petals. Fruit is globose, 3-4 mm. diam., smooth, succulent, black when ripe, like a peppercorn when dried, tipped with the persistent style[10]. **Fig 1.**

Distribution

E. ribes is highly restricted to hilly parts of India upto 1500 m. elevation from outer Himalayas to Western Ghats. It is also found in Sri Lanka, Singapore, South China and Malayan archipelago. It is distributed in moist deciduous forests of the Western Ghats of South India, Jammu & Kashmir, Arunachal Pradesh, Himachal Pradesh, Madhya Pradesh, Uttar Pradesh, Assam and Maharashtra[2,3,4,11].

Threat Status

Embelia ribes is considered as **Vulnerable (A1c, d)** in Karnataka and Tamil Nadu. It is of **Lower Risk Near Threatened** in Kerala[12].

Pharmacognosy

Physicochemical and Organoleptic Parameters Stem

Table 1: Physicochemical and Organoleptic Parameters Stem

Physicochemical Constants		Organoleptic Characters	
Parameters	**Values**	**Parameters**	**Values**
TA	1.7%	Taste	-
AIA	0.1%	Color	Light Brown
ASE	5.4%	Odour	Mild
WSE	7.6%	Texture	Fibrous

(TA - Total Ash; AIA - Acid Insoluble Ash; ASE - Alcohol Soluble Extractive; WSE - Water Soluble Extractive)

Figure1: *Embelia ribes* A twig with Inflorescence

TLC Finger Printing Profile

Prominent bands were observed with anisaldehyde spray and sulphuric acid spray at the Rf values 0.13, 0.33, 0.54, 0.97 and 0.37, 0.55, 0.98, respectively. All the other Rf values were given in Table. **Fig. 2.**

Anatomy of Stem

Fig. 3. T.S. of mature stem is somewhat circular in outline. The epidermis is single layered and is provided with unicellular glandular trichomes. Periderm is 5 to 6 layered which is followed by distinguishable cortical region of parenchymatous and schlerenchymatous tissues. Tannin cells are very prominent in parenchymatous region. Schizogenous cavities are also characteristic. Schlerenchymatous strand arches into the secondary phloem tissue. The secondary xylem and secondary phloem are produced by the cambium, as usual. Tannin cells are very common in the phloem tissue. Vessels are characteristically of large lumen. Medullary rays are thin walled and biseriate. Pith cells are parenchymatous[3].

Root

T.S. of mature root is somewhat circular in outline with single layered epidermis. Epidermis possesses multicellular

Table 2: Showing Rf values of bands with different detective reagents and UV light

Under Visible Light								
Rf Values	0.04	0.08	-	-	-	-	-	-
Sprayed with 10% H_2SO_4								
Rf Values	0.37	0.55	0.98	-	-	-	-	-
Sprayed with Anisaldehyde								
Rf Values	0.13	0.33	0.54	0.97	-	-	-	-
Under Short UV (254 nm)								
Rf Values	-	-	-	-	-	-	-	-
Under Long UV (366 nm)								
Rf Values	0.59	-	-	-	-	-	-	-

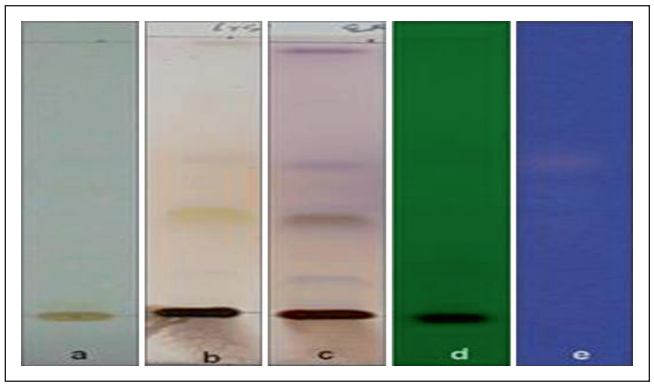

Figure 2: TLC fingerprinting profile of *Embelia ribes* stem (a) under visible light (b) sprayed with 10% H2SO4 (c) sprayed with anisaldehyde (d) observed under short UV 254nm (e) observed under long UV 366nm

and unicellular glandular trichomes. In mature root cork is about 15- 17 layered having schizogenous cavities. Cork is followed by a wavy schlerenchymatous ring. Beneath this, secondary vascular tissues are seen. Biseriate medullary rays with tannin cells are very conspicuous[3].

Petiole

T.S. of petiole is somewhat shield shaped in outline; vascular bundle is crescent shaped; epidermis is single layered with thin cuticle. Cortical region is parenchymatous. Schizogenous cavities are few when compared to that of stem. Tannin cells are very common[3].

Physicochemical and Organoleptic Parameters of Leaf

Table 3: Physicochemical and Organoleptic Parameters of Leaf

Physicochemical Constants		Organoleptic Characters	
Parameters	**Values**	**Parameters**	**Values**
TA	6.75%	Taste	Slightly sweetish
AIA	0.1%	Color	Green
ASE	9.2%	Odour	Mild
WSE	28.8%	Texture	Fibrous

(**TA** - Total Ash; **AIA** - Acid Insoluble Ash; **ASE** - Alcohol Soluble Extractive; **WSE** - Water Soluble Extractive)

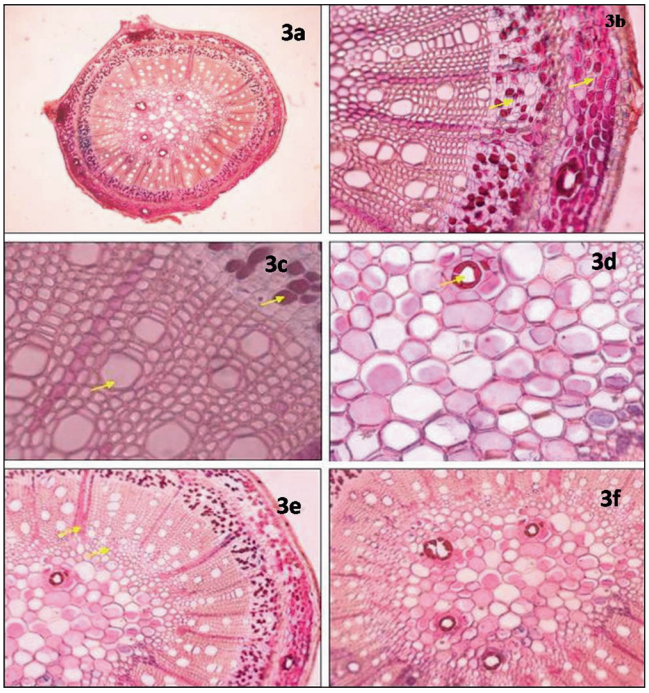

Figure 3: T. S. of *Embelia ribes* stem (a)whole view (b) cortex and cork (c) secondary xylem and tannin cells (d) schizogenous cavity (e) Medullary rays and secondary phloem (f) pith region

TLC Finger Printing Profile

Several bands were observed with anisaldehyde spray and long UV and Chlorophyll content is major contributor for the banding pattern. Rf values of bands were given in Table. **Fig.4.** After Sprayed with anisaldehyde total of eight distinct bands were observed

Anatomy of Leaf

Fig. 5. T.S. of leaf shows common dicotyledonous characters. Epidermis is single layered without any trichomes. Mesophyll consists of a single layered palisade, and spongy tissue with abundant intercellular spaces. Most of these cells are rich in tannin. Characteristic oil glands are very common in mesophyll especially near the midrib. Vascular bundle is broad 'C' shaped. Phloem fibers are very prominent. Stomata are of Ranunculaceous type[3].

Fruit (Macroscopic)

Fig. 6. Fruit brownish-black, globular 2-4 mm in diameter, warty surface with a beak like projection at apex, often short, thin pedicel and persistent calyx with usual1y 3 or 5 sepals present, pericarp brittle enclosing a single seed

Table 4: Showing Rf values of bands with different detective reagents and UV light after Sprayed with anisaldehyde for leaf

Under Visible Light								
Rf Values	0.08	0.39	0.78	-	-	-	-	-
Sprayed with 10% H_2SO_4								
Rf Values	0.09	0.38	0.54	0.97	-	-	-	-
Sprayed with Anisaldehyde								
Rf Values	0.12	0.38	0.54	0.61	0.7	0.76	0.9	0.98
Under Short UV (254 nm)								
Rf Values	0.05	0.22	0.95	-	-	-	-	-
Under Long UV (366 nm)								
Rf Values	0.03	0.19	0.34	0.44	0.59	-	-	-

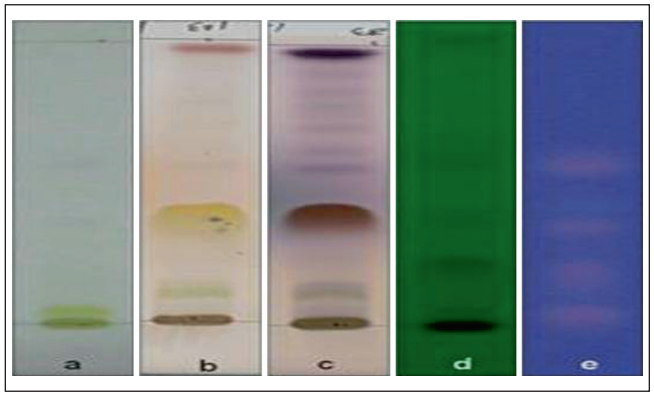

Figure 4: TLC fingerprinting profile *Embelia ribes* leaf (a) under visible light (b) sprayed with 10% H2SO4 (c) sprayed with anisaldehyde (d) observed under short UV 254nm (e) observed under long UV 366nm

covered by a thin membrane, entire seed, reddish and covered with yellowish spots (chitra tandula), odour slightly aromatic, taste, astringent[13].

Physicochemical and Organoleptic Parameters Fruit

Table 5: Physicochemical and Organoleptic Parameters Fruit

Physicochemical Constants			Organoleptic Characters	
Parameters	**Values**	**Limit**	**Parameters**	**Properties**
TA	6.2%	NA	Taste	Bitter
AIA	0.55%	NA	Color	Brown
ASE	1.0%	NA	Odour	Mild
WSE	10.0%	NA	Texture	Fibrous

(**TA** - Total Ash; **AIA** - Acid Insoluble Ash; **ASE** - Alcohol Soluble Extractive; **WSE** - Water Soluble Extractive)

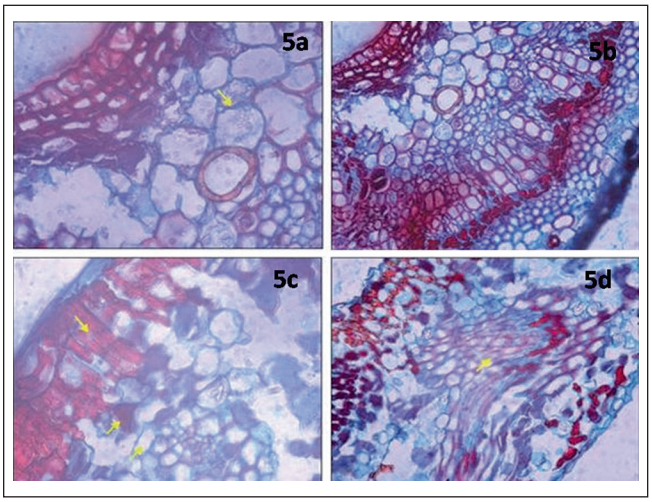

Figure 5: T. S. of *Embelia ribes* leaf (a) whole view (b) single layered epidermis (c) oil glands (d) C shaped vascular bundle

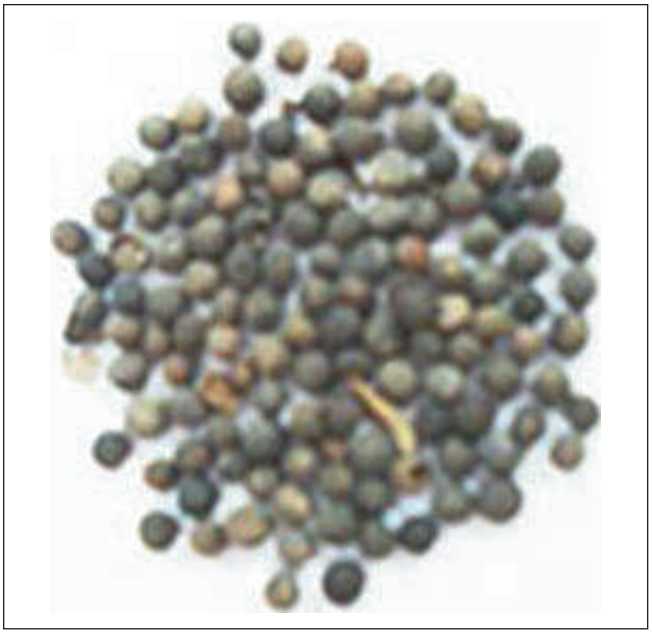

Figure 6: Fruits of *Embelia ribes*

TLC Finger Printing Profile Fruit

Prominent bands were observed with anisaldehyde spray and sulphuric acid spray at the Rf values 0.27, 0.91 and 0.32, 0.51, 0.56 respectively. One band is visible under long UV and short UV. **Fig. 7.**

Anatomy of Fruit

Fig. 8. Transverse section of fruit shows epicarp consisting of single row of tabular cells of epidermis, usually obliterated, (Fig 8b). In surface view cells rounded with wrinkled cuticle, Mesocarp consists of a number of layers of reddish-brown coloured cells and numerous (Fig 8c). Fibrovascular bundles and rarely a few prismatic crystals of calcium oxalate (Fig 8f, 8g & 8h). Inner part of mesocarp and endodermis composed of stone cells, endodermis consisting of single layered (Fig 8d). Thick-walled, large, palisade-like stone cells, seed coat composed of 2-3 layered reddish-brown coloured cells, (Fig 8e & 8f). Endosperm cells irregular in shape, thick-walled, containing fixed oil and proteinous mass.

Table 6: Showing Rf values of bands with different detective reagents and UV light Fruit

Under Visible Light								
Rf Values	-	-	-	-	-	-	-	-
Sprayed with 10% H_2SO_4								
Rf Values	0.14	0.22	0.37	0.42	0.58		-	-
Sprayed with Anisaldehyde								
Rf Values	0.22	0.31	0.41	0.5	0.58	-	-	-
Under Short UV (254 nm)								
Rf Values	0.41	-	-	-	-	-	-	-
Under Long UV (366 nm)								
Rf Values	0.13	0.27	-	-	-	-	-	-

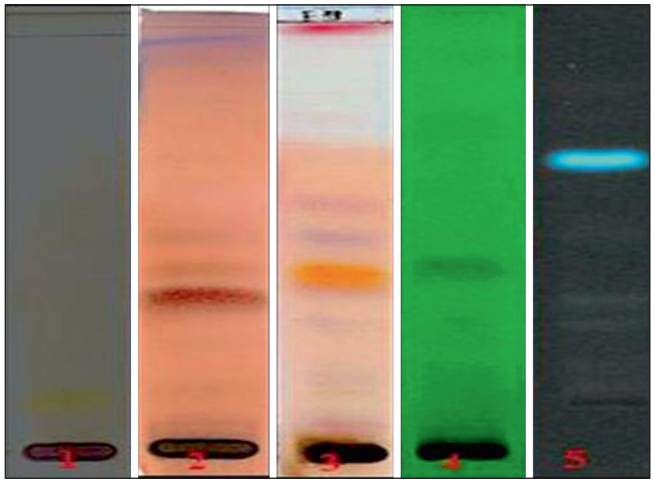

Figure 7: TLC fingerprinting profile *Embelia ribes* fruit (a) under visible light (b) sprayed with 10% H2SO4 (c) sprayed with anisaldehyde (d) observed under short UV 254nm (e) observed under long UV 366nm

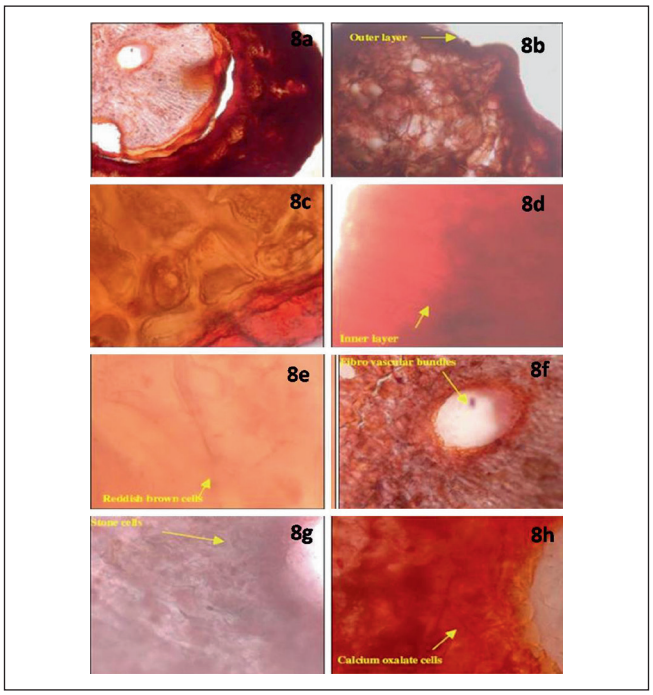

Figure 8: T. S. of Embelia ribes fruit (a) part view (b) outer layer (c) mesocarp region (d) inner mesocarp (e) reddiish brown color cells (f) fibro vascular bundles (g) stone cells (h) Calcium oxalate cells

Table 7: Microbial Limit Test Fruit

Raw herb sample	Total Aerobic Bacterial Count (TABC) (Cfu/gm)	Total Yeast And Mould Count (TYMC) (Cfu/gm)
Embelia ribes -Fruit	7.5×10^4	1.5×10^3

Microbial Limit Test Fruit

Results of microbial assay of *Embelia ribes -fruit* showed 75000 colonies for aerobic bacteria and 1500 colonies for yeast and moulds which is below the limits (TABC $\leq 1x10^7$; TYMC $\leq 1x10^5$) of international guidelines (Table 7)

Fruit Powder

Powder-Reddish, under microscope shows reddish parenchyma and stone cells[13].

Phytochemistry

Vilangin was isolated from the dry ripe berries of *E. ribes*. The structure was assigned as methylenebis (2, 5-dihydroxy-4-undecyl-3-6-benzoquinone)[14]. 3 new compounds identified as embelinol, embeliaribyl ester and embeliol were isolated from the seeds of *Embelia ribes* along with embelin[15]. Seeds of *Embelia ribes* showed the presence of Cr, K, Ca, Cu, Zn and Mn to be enough, with high carbohydrates but low position along with higher nutritive value[16].

An unusual nitrogen-containing 3-alkyl-1, 4-benzoquinone derivative, N- (3-carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1, 4-benzoquinone, and a gomphilactone derivative, 5,6-dihydroxy-7-tridecyl-3-[4-tridecyl-3-hydroxy-5-oxo-2(5H)-furylidene]-2-oxo-3(2H)-benzofuran were isolated from the roots of *E. ribes* using ethanolic extract[17].

Latha proposed an alternate way of extraction of embelin from *E. ribes*. Aromatic hydrotropes such as sodium n butyl benzene sulfonate (NaNBBS), and sodium cumene sulfonate (NaCS) were effective for selective extraction with a recovery of 95% from aqueous solution of hydrotropes with high purity[18]. Further a microwave assisted extraction procedure was developed where solvent selection, microwave energy input and solid loading were optimized. Maximum extraction was achieved in acetone and non-polar solvents such as hexane and dichloromethane were not effective for extraction of embelin[19].

Another unusual nitrogen-containing 3-alkyl-1, 4-benzoquinone, N-(3-carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1, 4-benzoquinone was isolated from *E. ribes*. The steps involved for isolation were a microwave-assisted combined Mitsunobu reaction-Claisen rearrangement to introduce the alkyl side chain into 2, 5-dimethoxyphenol, followed by alkene reduction, oxidation to the quinone, and sequential displacement of the methoxy groups with hydroxide and GABA tert-butyl ester[20].

High Performance Liquid Chromatography method was developed for determination of embelin in *Embelia ribes*. The Embelin content of 4.33% w/w was observed in *Embelia ribes*. The proposed method can be used for quantitative determination of embelin in Embelia plants[21].

HPLC analysis

The fruits of Embelia when extracted in 100% methanol for 30 minutes yielded 14.9-mg/gm dry weight of Embelin.

Uses

Traditional Uses

Fig. 7. The fruit is hot, dry, with a sharp bitter taste; it's a good appetizer; carminative, anthelmintic, alexiteric, laxative, alterative, cures tumor, ascites, bronchitis, mental diseases, dyspnoea, diseases of the heart, urinary discharges, jaundice, hemicrania, and worms in wounds[10]. Sushruta recommends the use along with liquorice for strengthening the body and to prevent the effect of age. The berries are used in the preparation of several compositions for ringworm and other skin diseases. The seeds are of high repute as anthelmintic particularly for tapeworms[10]. Pulp of the fruit is purgative. Fresh juice is cooling, diuretic and laxative[11].

Berries prevent flatulence and useful in dyspepsia. A paste of the seed is used against ringworm and other skin diseases.

Leaves along with ginger are used as a gargle for sore throat, aphthae and indolent ulcers of mouth[11]. Decoction of the root two or three times daily is effective against influenza[10]. Powder of dried root bark is a reputed remedy for toothache. For lung diseases like pneumonia paste of the bark is a valuable application for the chest. In headache paste is applied to the forehead made from berries of the plant and butter[80]. Vidanga taila made of *Croton tigilium*, *E. ribes* and carbonate of sodium is used for relieving headache or hemicranias[11].

The fruit is good for plethoric constitutions; analgesic, purgative, vulnerary, anthelmintic, cures bronchitis by thickening phlegm and drying; dries discharges from wounds, reddens the urine and removes bad humors from the body. It is considered to be attenuant and purgative of phlegmatic humors. Few berries are given to children with milk for children against flatulence[10].

Ayurvedic formulations

E. ribes is used in about 75 formulations where fruits are used in Ayurvedic preparations like Vidangarista, Vidanga Lauha, Vidangadi Lauha[13], Abhayarishtam, Ayaskrithi, Pippallyasavam, Anuthailam, Kachuradithailam[3].

Ethnobotanical Uses

According to Duke's ethnobotanical data *E. ribes* ethnobotanical properties are for vermifuge, alterative, anthelmintic, astringent, carminative, diuretic, stimulant, stomachic, taenifuge, tonic and tumor (Abdomen). It is also used in cough, diarrhea, chest disorders, fever, ringworm and other skin disorders[22].

Carbuncle

According to Siakia et.al., berries of *E. ribes* along with *Emblica officinalis, Piper longum, Terminalia bellerica* are mixed in equal amounts and the crushed form (powder) is added to honey and the concentrated honey is then applied on the infected carbuncle for relief[23].

Wound healing

According to Chopda and Mahajan fruits of *E. ribes* are used for wound healing in Jalgaon District of Maharashtra State[24].

Stomach Disorder

Tagin people of Arunachal Pradesh for stomach disorder use leaves and fruits of *E. ribes*[25].

Age related Cognitive disorders

Brahmarasayana one of the ayurvedic preparations used to treat age related cognitive disorders. *Emblica officinalis* is the main ingredient in the preparation along *E. ribes* and 16 others, which are also added to the mixture. This preparation is for a disease-free life with long lasting youth, great vigor and no dementia[26].

Cough & Cold

The roots of *E. ribes* grounded with lemon juice or buttermilk and 1-3 teaspoonful of juice is taken orally with sugar/jaggery, twice daily for 2 days to cure cough and cold in eastern region of Shimoga district[27].

Paralysis

For Paralysis the leaves of *E. ribes* along with roots of *Withania somnifera* (L.) Dun. and *Asparagus racemosus* Willd. and made into paste, which is taken orally with a cup of hot water, twice daily for 1 month[27].

Leucoderma

For leucoderma the mixture of *Withania somnifera* root, bark of *E. ribes*, leaves of *Plumbago zeylanica*, seeds of *Croton tiglium* and fruit pulp of *Cassia fistula* with cow's urine was applied on white patches for 2-3 months[28].

Anthelmintic

Powder of *Embelia ribes* fruits alone or mixed with powder of *Butea monosperma* with water or honey orally is used in traditional medicine as anthelmintic[29].

Pharmacological Uses

Lipid peroxidation

Hepatic antioxidant capacity of embelin (*Embelia ribes*) was tested in $CCl_{(4)}$-treated rats using different antioxidant tests where peroxidative damage was minimal in both liver and serum along with effectively inducing the antioxidant potential suggesting that that embelin acts as a natural antioxidant against hepatotoxicity induced in rats[30].

The aqueous extract of *Embelia ribes* fruit enhances the antioxidant defense against methionine induced hyperhomocysteinemia, hyperlipidaemia and oxidative stress in brain by decreasing the level of homocysteine, lactate dehydrogenase, total cholesterol, triglycerides, low density lipoprotein (LDL-C) and very low density lipoprotein (VLDL-C) and increased the high density lipoprotein (HDL-C) levels in serum. The extract also decreased lipid peroxides (LPO) levels with increase in glutathione (GSH) content in hyperhomocysteinimic rats[31].

Ethanol extract of *Embelia ribes* fruit significantly reversed the methionine increased homocysteine, lactate dehydrogenase, total cholesterol, triglycerides, and low-density lipoprotein levels in serum and lipid peroxides levels in heart homogenates with decrease in serum high-density lipoprotein and myocardial glutathione levels in pathogenic control rats. Thus indicating the effect of Embelia on

antihyperhomocysteinemic and lipid-lowering potential in hyperhomocysteinemic rats[32].

Lipid lowering and antioxidant potential of ethanolic extract of *E. ribes* Burm fruits was investigated in streptozotocin induced diabetes in rats. Twenty days of oral feeding of the extract resulted in significant decrease in blood glucose, serum total cholesterol, and triglycerides, and increase in HDL-cholesterol levels when compared to pathogenic diabetic rats. Further, the extract also lowered the liver and pancreas thiobarbituric acid-reactive substances (TBARSs) values providing the biochemical evidence for diabetic dyslipidemia of *E. ribes*[33].

In a comparative study between aqueous extract of *Tinospora cordifolia*, *Cyperus rotundus* and *Embelia ribes* on hyperlipidaemic rats the order of curative effects of *E. ribes* stood last when compared to *Tinospora cordifolia* and *Cyperus rotundus*[34].

Antihyperglycemic activity

Oral feeding of aqueous *E. ribes* extract to streptozotocin induced diabetic rats produced significant decrease in heart rate, systolic blood pressure, blood glucose, blood glycosylated hemoglobin, serum lactate dehydrogenase, creatine kinase and increase in blood glutathione levels. Further, significantly decreased the levels of pancreatic lipid peroxide and increased the levels of pancreatic superoxide dismutase, catalase and glutathione. Suggesting a significant blood glucose and blood pressure lowering potential by *E. ribes* along with enhancing endogenous antioxidant defense against free radicals produced under hyperglycemic conditions, thereby, seemingly protects the pancreatic beta cells against loss[35].

In another study Bhandari et.al., investigated the modulatory effect of chronic oral administration of *E. ribes* ethanolic extract on diabetes mellitus induced by a diabetogen, streptozotocin. Oral administration of the extract significantly reduced levels of blood glucose, glycated haemoglobin, heart rate and systolic blood pressure in rats[36].

Ethanolic extract of *Embelia ribes* enhances antioxidant defense against reactive oxygen species produced under hyperglycemic condition and thus protects beta-cells against loss, and exhibit antidiabetic property by decreasing blood glucose, blood glycosylated haemoglobin, serum lactate dehydrogenase, creatine kinase, pancreatic thiobarbituric acid-reactive substances (TBARS) levels and increase in blood superoxide dismutase, catalase and glutathione levels. There was expansion of islets when treated with test drug in diabetic rats[37].

Cardioprotective activity

Bhandari & Ansari demonstrated that ethanolic extract of *Embelia ribes* fruit attenuates isoproterenol induced oxidative stress in diabetic rats by enhancing cellular antioxidant defense through significant increase in heart rate, blood glutathione, serum lactate dehydrogenase, and myocardial endogenous antioxidant levels along with decrease in systolic blood pressure, blood glucose, HbA1C, serum creatine kinase, and myocardial thiobarbituric acid reactive substances levels[38].

In another trial with the same ethanol extract decreased the elevated levels of lactate dehydrogenase and creatine kinase in serum and myocardial thiobarbituric acid reactive substances and increased reduced levels of glutathione, superoxide dismutase and catalase in heart homogenates in isoproterenol induced myocardial infarction in albino rats. Histopathological studies observed a marked protection by the extract in myocardial necrotic damage[39].

In another study aqueous extract of fruits of *Embelia ribes* decreased significantly the heart rate, systolic blood pressure, increased levels of serum lactate dehydrogenase, serum creatine kinase and myocardial lipid peroxides and increased significantly the myocardial endogenous antioxidants like glutathione, superoxide dismutase and catalase levels in isoproterenol treated rats. The results were supplemented with histopathological examination of rat's heart sections to confirm the myocardial injury[40].

Neuroprotective activity

Aqueous extract of *Embelia ribes* pretreatment ameliorates cerebral ischemia/reperfusion injury and enhances the antioxidant defense against middle cerebral artery occlusion-induced cerebral infarction in rats by significant increase in the post stroke grip strength activity. Further reversed the levels/activities of thiobarbituric acid reactive substances; enhanced glutathione; glutathione peroxidase; glutathione reductase; and, glutathione-S-transferase and also resulted in decreased cerebral infarct area, as compared to the ischemic group, thus exhibits neuroprotective property[41].

Administration of ethanolic *E. ribes* extract orally to methionine induced hyperhomocysteinemic rats produced a significant decrease in the levels of homocysteine, LDH, total cholesterol, triglycerides, in serum and LPO levels in brain homogenates with significant increase in serum HDL-C levels and GSH content in brain homogenates, when compared with pathogenic control rats. Degenerative changes of neuronal cells in methionine treated rats were minimized to near normal morphology as evident by histopathological examination[42].

Chronic pretreatment with ethanolic *E. ribes* extract enhances the antioxidant defense against middle cerebral artery occlusion induced focal cerebral ischemia in rats and exhibits neuroprotective activity by significant increase in the grip

strength activity, and glutathione, glutathione peroxidase, glutathione reductase and glutathione-S-transferase levels in hippocampus and frontal cortex with significant decrease in lactate dehydrogenase levels in serum and thiobarbituric acid reactive substance levels in hippocampus and frontal cortex[43].

Antioxidant Activity

Embelin (from *Embelia ribes*) is a component of herbal drugs and possess wide range of medicinal properties. It has been found to scavenge DPPH radical and inhibit hydroxyl radical induced deoxyribose degradation. It was also found to inhibit lipid peroxidation and restore impaired Manganese-superoxide dismutase in rat liver mitochondria. Further, kinetics and mechanism of the reactions of embelin with hydroxyl, one-electron oxidizing, and organo-haloperoxyl and thiyl radicals were studied using nanosecond pulse radiolysis technique[44].

Hepatoprotective Activity

Embelia ribes commonly known as vidanga has been reported to be useful in jaundice. It is a constituent of various formulations marketed for liver ailments. In a hepatoprotective study the ethanolic extract of *Embelia ribes* on paracetamol induced liver cell damage was studied using mice as experimental animals. The mice treated with *Embelia ribes* extract showed a dose dependent fall in the serum glutamate pyruvate transaminase (SGPT) levels. Histopathology of liver revealed normal livers in a dose dependant manner[45].

Antitumor activity

Embelin was found to exhibit significant antitumor activity in methylcholan-threne-induced fibrosarcoma in albino rats along with enhancing their survival time. It also had an appreciable action on pain and inflammation. The changes in DNA, RNA and protein levels in tumor bearing control and drug treated animals were also studied[46].

Chitra et.al. In an in vitro study assessed drug induced cell toxicity using a rapid technique in a fibrosarcoma cell line where the cells were inoculated with increasing concentrations of embelin along with [3H]-thymidine, where thymidine uptake was decreased by embelin in lipid peroxidation, and glutathione levels dose dependently[47].

Chemopreventive effects of embelin against N-nitrosodiethylamine/Phenobarbital-induced hepatocarcinogenesis in Wistar rats were studied where embelin prevented the induction of hepatic hyper plastic nodules, body weight loss, increase in the levels of hepatic diagnostic markers, and hypoproteinemia[48].

In a trial on carbohydrate moieties of glycoprotein in plasma, liver and kidney of tumor bearing rats continuous administration of embelin lowered the values of total hexose, hexosamine and sialic acid to near normal indicating an antitumor activity[49].

1, 4-benzoquinone derivatives 5- O-ethylembelin and 5-O-methylembelin showed antiproliferative activity against a panel of human tumor cell lines upon comparison to normal marsupial kidney cells (PtK2). They inhibited multiplication of HL-60 cells in the G (0)/G (1) phase of the cell cycle. When HeLa cells, were exposed to 100 μM of 1, 4-benzoquinone derivatives 5- O-ethylembelin and 5- O-methylembelin for 6 h complete disassembly of the microtubule network and an increased number of cells blocked in mitotic stages were observed. Apoptosis in HL-60 cells was observed when treated with 10 μM of 1, 4-benzoquinone derivatives 5- O-ethylembelin and 5-O-methylembelin for 24 h. Suggesting both 1, 4-benzoquinone derivatives 5- O-ethylembelin and 5- O-methylembelin are promising novel antimitotic and anticancer molecules targeting microtubular proteins[50].

Chen et.al., had previously identified that embelin as an inhibitor of XIAP through computational structure-based database screening. Further efforts led to the identification of new and more potent inhibitors like compound 6g has Ki value of 180 nM binding to XIAP BIR3, in competitive binding assay and represents a promising lead compound for further optimization[51].

Embelin, identified primarily from the *Embelia ribes* plant, is a compound shown to exhibit chemopreventive, anti-inflammatory, and apoptotic activities through an unknown mechanism which was demonstrated when it inhibited tumor necrosis factor, alpha-induced NF-kappaB activation, the TNF α-induced activation of the inhibitory subunit of NF-kappaBα kinase, IkappaBα phosphorylation, IkappaBα degradation, p65 phosphorylation, nuclear translocation. It also suppressed NF-kappaB-dependent reporter gene transcription, TNF receptor-1, TNFR1-associated death domain protein, TNFR-associated factor-2, NF-kappaB-inducing kinase, and IkappaBalpha kinase. Furthermore, embelin down-regulated gene products involved in cell survival, proliferation, invasion, and metastasis of the tumor. This down-regulation was associated with enhanced apoptosis by cytokine and chemotherapeutic agents. In addition, NF-kappaB activated by diverse stimuli was suppressed[52].

Embelin inhibits chemical carcinogen-induced colon carcinogenesis, which is partially dependent on presence of functional PPARgamma, was proved by Dai et.al., where it inhibited proliferation and induced apoptosis in HCT116 cells with marked up-regulation of PPARgamma. In addition, it significantly inhibited expressions of survivin, cyclin D1, and c-Myc, which were partially dependent on

PPARgamma. Embelin significantly reduced incidence of colon cancer in PPARgamma (+/+) mice but not in PPARgamma (+/-) mice. Embelin inhibited NF-kappaB activity in PPARgamma (+/+) mice but marginally in PPARgamma (+/-) mice. Thus, reduced expression of PPARgamma significantly which sensitizes colonic tissues to the carcinogenic effect of 1, 2-dimethylhydrazine dihydrochloride[53].

Effect of bicalutamide and embelin on growth of prostate cancer cells in vitro and in vivo was carried out. Embelin induced caspase 3 and 9 activation in LNCaP and C4-2 cells by decreasing XIAP expression and was more potent than bicalutamide in killing prostate tumor cells irrespective of their androgen status. According to isobologram analysis combination of bicalutamide and embelin was synergistic for C4-2 but additive and slightly antagonistic for LNCaP cells. Increase in aqueous solubility of drugs resulted in Micellar formulation. Tumor growth was effectively regressed upon treatment with bicalutamide, but tumor response stopped after prolonged treatment of bicalutamide and began to grow. Sequential treatment with XIAP inhibitor embelin resulted in regression of these hormone refractory tumors[54].

Embelin has been used as an anticancer agent in therapeutic studies. In one of the study embelin along with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or the combination of both were tried against glioblastoma cells and human astrocytes. It broadly sensitized malignant glioma cells to TRAIL-mediated apoptosis. Combined treatment augmented activation of initiator caspases-8/-9 and effector caspases-3/-7. Further Embelin alone down-regulated expression of long- and short-isoform of c-FLIP and forced expression of short isoform of c-FLIP attenuated apoptosis. It did not modulate mRNA levels of c-FLIP (S), suggesting that Embelin modulates expression of c-FLIP in a posttranscriptional manner[55].

Wound healing activity

Wound healing activity by excision, incision and dead space wound models on Swiss Albino Rats was carried out where ethanol extract of the leaves of *Embelia ribes* Burm. and its isolated quinone compound embelin were screened. Significant wound healing activity was observed in both ethanol crude extract and embelin treated groups. In embelin treated groups, epithelialization of the incision wound was faster with a high rate of wound contraction. The tensile strength of the incision wound was significantly increased than the ethanol extract. In dead space wound model also the weight of the granulation was increased indicating increase in collagenation. The histological examination of the granulation tissue of embelin treated group showed increased cross-linking of collagen fibers and absence of monocytes[56].

Acetylcholinesterase activity

Dysfunction of cholinergic neurotransmission in the brain contributes to the salient cognitive decline in Alzheimer's disease. Loss of cholinergic cells, particularly in the basal forebrain, is accompanied by loss of the neurotransmitter acetylcholine. One of the most accepted strategies in Alzheimer's disease treatment is the use of cholinesterase inhibitors. Their clinical efficacy is thought to result from prolonging the half-life of acetylcholine through inhibition of AChE. The half-life of acetylcholine inhibition of methanolic extract of *E. ribes* root was 23.04 µg/ml, which partially substantiates the traditional use of *E. ribes* for improvement of cognition[57].

Antifertility activity

Altered metabolic function was observed along with significant rise in levels of acid and alkaline phosphatases of testis and prostate when embelin was administered along with *Vinca rosea* to male albino rats[58].

Treatment with 50% ethanol and benzene extracts of *Embelia ribes* Burm. increased the glycogen, protein and non-protein nitrogen contents in the uterus of normal and ovariectomized rats. The effect is statistically significant at low dose level but decreases as the dose is increased. Higher dose of benzene extract is toxic[59].

Long-term metabolic effect of embelin on the testes of adult male dogs was evaluated by feeding them with embelin for 100 days. A three tiered finding containing histology, tissue biochemistry and blood/serum profile of dogs treated with embelin showed that 100 days therapy inhibits spermatogenesis, loss in weights of testes and spermatogenic elements and epididymides was devoid of spermatozoa but functional morphology remained unaltered. The 250 days of recovery period brought about normal spermiogenesis with all 1-8-cell stages and epididymal milieu showed functional physiology. This proved that 250 days recovery period restored all contraception like activity of embelin. Sexual potency and libido of the animals were not altered. Therefore a reversible male contraception with the help of embelin looks promising[60].

Traditional medicinal plants having contraceptive efficacy were searched, identified and collected throughout India. Contraceptive properties were studied in rats, mice, and hamsters. Significant contraceptive effects were seen after administration of *Crotalaria juncea* Linn, *Verbena hybrida* Linn, *Verbena bonariensis* Linn, and *Embelia ribes* Burm, *Artabotrys odoratissimus* Linn., and *Pueraria tuberosa*. Biological property of *Embelia ribes* Burm and *Artabotrys odoratissimus* Linn were interesting but had strong toxic effects[61].

Antifertility effects of *E. ribes* berries, its petroleum ether, methanol, chloroform and benzene extracts, embelin and the putative active principal were reported. The berries showed 62% antifertility effect when incorporated into the diet at 10 & 20% dose levels, which was due to prolonged diestrous phase within 2 weeks of treatment. In serially hot-extracted petroleum ether and methanol extract cyclicity was affected and prevented pregnancy in 75% of treated rats. Benzene extract showed 51%, whereas chloroform extracts showed 37% antifertility activity. These doses were all administered post-coitally, from Day 1-7 of pregnancy. Antifertility effect was assessed by number of implantation sites post treatment[62].

Semen analysis and hormonal levels in Bonnet macaques after three months of administration of *E. ribes* berries did not affect spermatogenesis but reduced circulating testosterone level[63].

Antiandrogenic activity of Embelin extracted from *Embelia ribes* Burm. berries was observed where the berries, altered the testicular histology and glycogen, gametogenic counts and accessory sex gland fructose at different dosage levels[64].

Estrogenic mode of action of *E. ribes* was observed when the 50% ethanolic and benzene extract of dried berries increased the intensity of reaction for alkaline phosphatase in luminal and glandular epithelium[65].

Daily subcutaneous administration of embelin, the active principle of the seeds of *Embelia ribes* Burm, revealed inhibition of epididymal motile sperm count, fertility parameters such as pregnancy attainment and litter size, and the activities of the enzymes of glycolysis and energy metabolism. Further addition of embelin to epididymal sperm suspension caused inhibition of spermatozoal motility and the activities of the enzymes of carbohydrate metabolism. Histological observations showed that both invivo and Invitro treatment caused morphological changes in spermatozoa such as, decapitation of the spermatozoal head, discontinuity of the outer membranous sheath in the mid-piece and the tail region, and alteration in the shape of the cytoplasmic droplet in the tail, displaying the antispermatogenic effect of embelin on male albino rats[66].

It is suggested that uterine peroxidase assay can be utilized as a biochemical parameter in the screening of new antifertility agents for their estrogenic/antiestrogenic properties. In one of the trial uterotropic and uterine peroxidase activities in ovariectomized rats were highly correlated in response to treatment with embelin[67].

Male fertility regulating potential of embelin was investigated in rabbits. There was a marked reduction of testosterone concentrations within two days of administration and up to 90% reduction by the 6th day. Luteinising hormone showed a corresponding rise with the falling testosterone levels and also there was rapid increase in progesterone levels. The concentrations of progesterone and luteinising hormone declined when dosage was stopped. This suggests that embelin disrupts production of testosterone at the testicular level[68].

In a trial on most effective and convenient route of delivery of embelin was carried out on plasma testosterone levels of sexually mature male white New Zealand rabbits. In which oral administration offered the best and effective method of drug delivery system[69].

Embelin has a role to play in reproductive functions in female rats as it disrupted oestrous cycles and there was significant depression in plasma oestradiol and progesterone. In vitro studies isolated mixed ovarian cells from embelin treated rats produced significantly less progesterone and estradiol than controls. Thus confirming that embelin interferes with reproductive functions in female rats by suppressing ovarian production of sex steroid hormones[70].

The developmental toxicity of in utero exposure to pippaliyadi yoga or pippaliyadi vati an ayurvedic contraceptive used in India since ancient times was studied by administering low and very high doses to gravid females from day 6 to day 16 of gestation. Pippaliyadi vati did not have any adverse developmental effects with low doses; however, with higher dose decrease in body weight of the pups was observed. The reproductive performance of the progeny born to mothers treated with pippaliyadi vati was not significantly affected. The present study suggests that *in utero* exposure to pippaliyadi does not have any adverse effect on the postnatal development and reproductive performance of the F_1 progeny[71].

Analgesic activity

Embelin and all its disalts showed analgesic activity in which 2:5 disobutyl amine embelin showed maximum action. The effect was observed after intraperitoneal administration but not after subcutaneous, intramuscular or oral administration. Analgesic effect was seen in dogs and cats after intravenous injection also. Embelin and 2:5 isobutyl amine embelin also exhibited antipyretic and anti-inflammatory activities[72].

Embelin derived from *E. ribes* was studied for its analgesic effects in rats and mice. It was effective by oral, Intra muscular and ICV routes and could be compared with morphine. The potassium embellate acts centrally to produce analgesia; its effect is not antagonized by naloxone indicating a different central site of action. High oral efficacy and

non-narcotic properties of the test drug make it more acceptable than morphine. In addition, lack of any adverse effects, high therapeutic index and absence of abstinence syndrome confers a long-term safety on potassium embelate for use as an analgesic[73]. In another study Zutshi et.al., observed that the analgesic property of potassium embelate may be due to the involvement of mu and kappa binding site in the brain[74].

In a study on analgesic activity of embelin it was revealed that there are naloxone resistant specific binding sites for potassium embelate in the spinal cord through which the antinociceptive response is modulated. Mono and dipotassium salts of embelin, displayed higher analgesic activity in visceral evoked responses when compared with thermal evoked responses. It is suggested that potassium embelate has strong affinity for kappa type of opiate receptors[75].

The effect of potassium embelate on neurotransmitter content in cerebrospinal fluid of dog was observed where the drug significantly affected levels of noradrenaline and acetylcholinesterase activity[76].

In a pahramacokinetic study by Zushi et.al., revealed that biexponential kinetic pattern was followed by the compound. Bioavailability was complete and fast. The disposition half-life is 9.5 h on intravenous and 11 h on oral administration. High concentrations of the drug were found in brain between 0.25 and 2 h, which is in agreement with its pharmacological action. The kidney plays a major role in the excretion of the drug[77].

Anthelmintic activity

Seed oil of *Embelia ribes* was investigated for its anthelmintic property against *Pheritima posthuma*. Three concentrations of oil were studied in the bioassay, which involved determination of time of paralysis and time of death of the worm. The oil exhibited moderate to significant anthelmintic activity. When compared with other plants like *Gynandropsis gynandra*, *Impatiens balsamina*, *Celastrus paniculata* and *Mucuna pruriens E. ribes* had the best anthelmintic activity[78].

Ethanolic extract of *E. ribes* fruits showed an anthelmintic efficacy of up to 93% against gastrointestinal nematodal larvae *Haemonchus contortus*[79].

Antinematodal activity of mixed preparation of *Vernonia anthelmintica* seed and *Embelia ribes* fruit was studied in goats with water, methanol extract and powder, which was compared with morantel tartarate. Egg per gram (EPG) counts was made in the faeces before and on the 3rd, 10th and 15th days of treatment. The data of 15th day of administration showed that 2 g/kg of powder, its equivalent amount of methanol extract and 0.01 g/kg of morantel tartarate are equally effective and safe in treating natural gastrointestinal nematode infection of the local goats[80].

Antibacterial activity

Embelin, isolated from *E. ribes*, exhibited significant inhibition against *Staphylococcus aureus, Streptococcus pyogenes, Shigella flexneri, S. sonnei* and *Pseudomonas aeruginosa* and moderate activity against *Salmonella typhi, S. boydii* and *Proteus mirabilis*[81].

Moderate antibacterial activity was shown by methanol and aqueous extract of *Embelia ribes* against multi-drug resistant *Salmonella typhi*[32].

Clinical trial on contraceptive activity

A clinical trial was conducted on 48 fertile women (age 26-42 yrs) for 1 year covering 538 menstrual cycles with indigenous oral contraceptive Maswin (*Embelia ribes*). I tablet (400 mg extract) was taken each morning for 10 days beginning on the fifth day of menstruation; total dosage was 10 tablets for 1 menstrual cycle. No pregnancies, side effects, or toxic symptoms occurred. All expressed a sense of well-being and were eager to continue usage of Maswin. *Embelia ribes* antagonizes the effects of estrogen on the uterus so the uterus is not in the necessary condition to accept fertilized ovum, so pregnancy does not take place even though ovulation and fertilization have occurred[83].

Safety

Potassium embelate, from *Embelia ribes* Burm. was subjected to sub-acute, chronic, reproductive toxicity testing and teratological investigations in laboratory animals (mice, rats and monkeys). Adverse effects were not observed in the animals indicating that potassium embelate is a safe compound[84].

Toxicity studies

The alcoholic and aqueous extracts of the berries of *E. ribes* on rats did not reveal any toxic effect on male reproductive organs[85].

In a study conducted by Low et.al., retinal pathology and defects in visual behavior in chicks treated with *Embelia ribes* (Enkoko), *Hagenia abyssinica* (Kosso), or embelin, a crystalline extract of *E. ribes* was observed. The chicks fed with high doses of the anthelmintics significantly reduced the ability of chicks to detect a moving bead introduced into peripheral field of vision. Degree of constriction of the visual field for detection was dose dependent. Performance on a visual discrimination task, which required discrimination of feed grains from pebbles, was also impaired. The visual deficits observed in Enkoko-treated

animals were mimicked by embelin, which suggests embelin may be responsible for visual defects. Anatomical evidence of degeneration of ganglion cells was found in retinae exposed to high doses. However, no retinal lesions were detected in animals following treatment with low doses[86].

In a short-term toxicity study wet weight and biochemical constituents of the adrenals and activity of acid and alkaline phosphatase in kidney and adrenal showed a remarkable increase, whereas no changes were observed in weight of liver, kidney and spleen and in biochemical constituents such as protein and glycogen. Embelin intake for 6 weeks caused severe pathological changes in liver and kidney like disintegration, necrotic changes and perinuclear vacuolation[87].

In an embryotoxicity and teratogenicity studies of an ayurvedic contraceptive--pippaliyadi vati, containing equal parts of powdered seeds or fruit berries of *Embelia ribes*, fruit of *Piper longum* and borax powder foetuses of mothers fed with pippaliyadi vati had low birth weights and were smaller in length. The mothers gained less weight during gestation. When developmental defects of soft tissues and skeletons were analyzed there were instances of herniation of the intestines into the umbilical cord in foetuses of mothers[88].

Miscellaneous

In a study to develop tablet formulation of embelin co-crystallized lactose-microcrystalline cellulose was the best diluent and alcoholic polyvinyl pyrrolidone proved to be the best binder. Solubility study revealed that it has optimum solubility in phosphate buffer of pH 8 and in 2% aqueous sodium lauryl sulfate solution and incorporation of 10% v/v ethanol to phosphate buffer of 7.4 pH enhanced the solubility of embelin[89].

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Development of Fingerprinting Methods of Balacaturbhadrika Churna: An Ayurvedic Formulation

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ABSTRACT

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

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

INTRODUCTION

In the past decade, there has been renewed attention and interest in the use of traditional medicine (Ayurveda, Yoga, Naturopathy, Unani, Siddha and Homeopathy) in India and globally. Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain[1]. The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them[2-3]. The utilization of plants for medicinal purposes in India has long history, and the proportion of medicinal plants is the highest proportion of plants known for their medicinal purposes in any country of the world for the existing flora of the respective country. Medicinal plants are essential natural resource which constitutes one of the potential sources of new products and bioactive compounds for drug development[4].

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It is estimated that 80% of the population in rural India use medicinal plants to meet primary health care needs[5]. Continuous erosion in the traditional knowledge of many valuable plants for medicine in the past and the renewal interest currently, the need existed to review the valuable knowledge with the expectation of developing the medicinal plants sector[6]. Under the parasol of traditional medicine systems the Ayurvedic system of medicine also gaining global acceptance due to its amazing clinical efficiency. While Ayurvedic systems of medicines have long been used, there is negligible documented evidence regarding its safety and effectiveness. The lack of evaluation has, in turn, slowed down the development of regulations and legislation. Recently Good Manufacturing Practices (GMP) rules for Ayurvedic medicines to ensure the quality of the manufactured drugs and gain credibility to make them acceptable globally. The Drugs and Cosmetic Act 1940 controls the standards of manufacturing, sale and distribution of Ayurvedic drugs[7]. Balacaturbhadrika churna is a fine powder form, which is widely used in Diarrhoea, Fever, Cough and Asthma at dose of 500 mg to 1 gm/day[9]. It is composed of Ghana (musta), Krsna (pippali), Aruna (ativisa) and Sringi (karkatasringi). All the ingredients are firstly powdered separately and mixed together.

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

MATERIALS AND METHODS

Materials

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

Preparation of formulation

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

Determination of ash values

Total ash

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

Acid-insoluble ash

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

Extractive values

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

Qualitative phytochemical studies

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

Bulk density

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

Tap density

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

Angle of repose

A glass funnel is held in place with a clamp on ring support over a glass plate. The glass plate is placed on a micro-lab jack. Approximately 100 g of powder is transferred in to the funnel (that was previously passed through a number 10 mesh size), keeping the orifice of funnel blocked by the thumb. As the thumb is removed, the lab – jack is adjusted

so as to lower the plate and maintain about 6.4 mm gap between the bottom of funnel stem and top of the powder pile. When the powder is emptied from the funnel, the angle of the heap to the horizontal plane is measured with a protector[12]. Measure the height of the pile (h) and the radius of the base(r) with the ruler. The angle of repose is thus estimated by following formula.

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

Hausner Ratio

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

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

Carr index

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

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

Preparation of calibration curve for Piperine

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

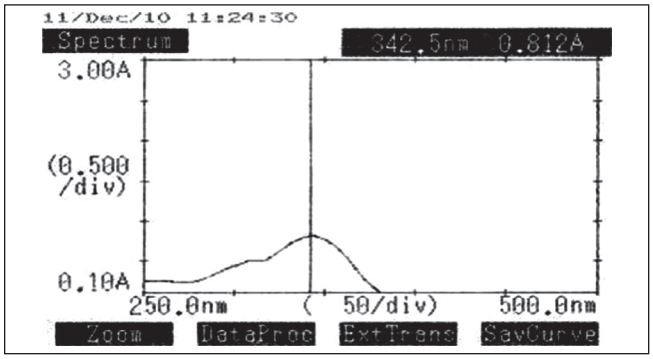

Figure 1: UV scan of piperine in ethanol

RESULTS AND DISCUSSION

Physicochemical properties

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

Phytochemical analysis

Results of the phytochemical screening of the raw materials, lab formulation and marketed formulation of

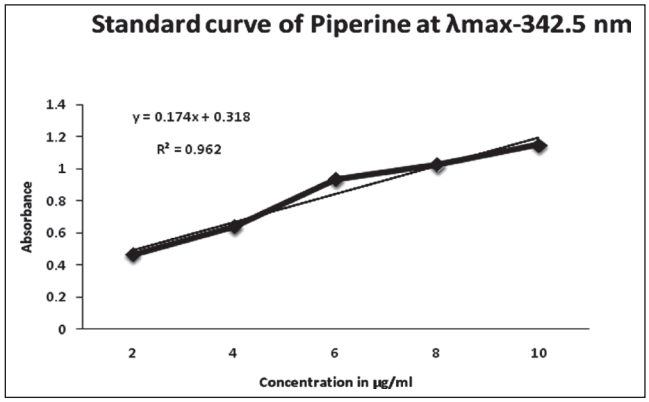

Figure 2: Standard curve of piperine in ethanol at 342.5 nm

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

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

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

Balacaturbhadrika churna are concluded in Table-II. One notable difference as a result of the methods of extraction is the possibility that the alkaloids in *Piper longum* and *Pistacia integerrima* are more soluble in ethanol, the reason why the presence of that group was not detectable in the aqueous extract. Furthermore, where more than one test was conducted for the detection of a chemical group such as the alkaloids, no differences in the results were observed for the different tests.

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

Determination of Ash value

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

Determination of extractive values

Alcohol-soluble and water soluble extractive values of ingredients and formulation are depicted in Table-IV, which shows 39.294 ± 2.226 and 30.662 ± 0.472 alcohol-soluble extractive value for lab and marketed formulation respectively which is higher than water soluble extractive value of both formulations. Higher ethanol-soluble extractive value implies that ethanol is a better solvent of extraction for the formulation than water.

Spectrophotometric analysis of piperine

The determination of formulations was carried out through UV spectrophotometer at 342.5 nm for piperine. The absorbance characteristics show that piperine follow Beer Lambert's law within the concentration range 2-10 µg/ml at the λ-max of 342.5 nm. The estimation of piperine content of the balchaturbhadrika churna and powder of

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

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

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

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

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

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

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

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

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

Table 5: Spectrophotometric determination of piperine content

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

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

Piper longum (Pippali) was carried out separately. The concentration of piperine content in raw material was found to be 0.695 ± 0.012 w/w in Piper longum. The content of piperine in different batches of balchaturbhadrika churna was found to be 0.392 ± 0.030 %, 0.363 ± 0.001 %, 0.382 ± 0.004 % and 0.233 ± 0.002 % w/w respectively for lab formulation (LF-1, LF-2, LF-3) and marketed formulation (MF) (Table-V). The developed method was found to be reliable, accurate, precise and sensitive.

CONCLUSION

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

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Pharmacognostic Studies and Establishment of Quality Parameters for *Albizia altissimum* (Hook.f) Hutch et Dandy Leaf

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ABSTRACT

Introduction: A large population of Nigerian like in other part of the developing world depend on the use of plants to meet their health needs and to address the perceive inadequacies associated with this form of therapy it is essentials that quality standard are established for plants used in traditional therapy. This study was carried out for *Albizia altissimum,*(Hook.f) Hutch et Dandy a plant widely used in the Niger Delta Region of Nigeria for mental disorder among many other uses. **Methods:** The World Health Organization (W.H.O) guidelines for the establishment of quality parameter for medicinal plants were used to carry out all the determinations-.microscopic, macroscopic and quantitative parameters. **Results:** Transverse section of the leaf showed a single layer epidermis with cuticle and trichomes, mesophyll divided into palisade and spongy mesophyll as well as druse calcium oxalate crystals along the vein while physicochemical parameters such total ash, water soluble ash and acid insoluble ash were also determined. **Conclusion:** The parameters determined above can serve as a platform for the preparation of a monograph for the leaf of *A. altissimum.*

Key words: Macroscopic and Microscopic Characteristics, Medicinal plants, Standardization

INTRODUCTION

Plants have always been a part of mankind`s healthcare system and plants in the form of herbal medicine plays an important role in the healthcare system of most developing countries, Nigeria inclusive. The use of plants, parts of plants and isolated photochemical for the prevention and treatment of various health ailments has been in practice from time immemorial.[1,2] The World Health Organization states that approximately 85-90% of the world`s population consumes traditional medicines and nearly 80% of African and Asian populations depends on this type of medicines for their healthcare and about 85% of traditional medicines involves the use of plants[3,4] While there is an increase usage of herbal drugs throughout the world, reports on side effects and adulteration of herbal drugs have raised concern on their wide use and thus affecting their commercialization.

Adulteration or substitution involves intentional replacement with another plant species or intentional addition of foreign substances to increase the weight or potency of the product or to decrease its cost. The use of fake or wrong herbs has generated serious questions about the safety and efficacy of herbal drugs .[2]] To ensure reproducible quality of herbal products proper control of starting materials is of utmost importance and the first step towards ensuring quality of starting material is authentication. Thus, in recent years there has been a rapid increase in the standardization of selected medicinal plants of potential therapeutic significance. Pharmacognostic studes are pivotal in herbal sciences as it ensures plant identity and prevents adulterations[5] hence for any drugs to be continually produced to the same standard of safety and efficacy all the time, it is necessary to establish quality control parameters that must be met at all times[6] and this is the major reason why quality standard must be established for medicinal plants used in traditional medicine with proven efficacy in the treatment of various diseases of man.

The Plant Albizia altissimum (Hook f) Hutch and Dandy

The plant is a deciduous tree that grows to about 15 m in height and about 25 cm in diameter and it s common in the riverine forest and secondary jungle from Sierra Leone

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to West Cameroon extending to Sudan, Uganda and Angola. The leaves, stem, root and fruits have various uses in traditional medicine such as in the treatment of snake bite, use as fish poison, treatment of tooth and stomach ache as well as in the treatment of mental illness.[7] The bark is used in traditional medicine in Sierra Leone and DR Congo; a decoction is used as an antidote to treat toothache and stomach-ache, and against pulmonary infections, and externally to treat sores. The bark also serves as fish poison. Scraped inner bark beaten up in water is used as soap to wash clothes.[8] In DR Congo a leaf decoction is used in a vapor bath to treat colds. Burned leaves are applied to snakebites. The fruits are used for tanning and dyeing, and to prepare ink and soap. The fruit pulp and the seeds are edible. In Nigeria fermented seeds called 'oso' are used as condiment in soups.[9] Scientific studies on the 50% Ethanol extract of the leaves has justified the traditional uses of the plants in treatment of mental disorder as it showed central nervous system depressant effect in mice.[10]

MATERIALS AND METHODS

The leaves for the study were collected from the back of Medicinal Plant garden of the Department of Pharmacognosy and Herbal Medicine, Faculty of Pharmacy of Niger Delta University Wilberforce Island Bayelsa State South-South Nigeria. It was authenticated by Mr. Benjamin Daramola a retired Taxonomist from the Forestry Research Institute of Nigeria and an herbarium specimen was kept in the Department of Pharmacognosy and Herbal Medicine Herbarium with Voucher Number PCGH 001. The leaves were air dried and ground to powder with a grinding machine and stored until needed.

Pharmacognostic studies

The World Head Organization guidelines[11] for quality control methods for medicinal plants were used for all the parameters determined. Fresh leaves of the plant were used to prepare surface preparation. The method as described by Bitasta and Swati[12] was used for the fluorescence analysis of the powder.[12]

RESULTS

Table 1: Results of some physiochemical parameters

Parameters	mg/g
Moisture content	482.50
Total ash	30.00
Acid insoluble ash	0.027
Water soluble ash	0.051
Ethanol soluble extractive value (cold extraction)	150.00
Ethanol soluble extractive value (hot ethanol)	182.50
Water soluble extractive value (cold extraction)	130.00
Water soluble extraction value (hot extraction)	162.00.

Table 2: Mean values of stomata index

Stomata index	Value
	7.143-**8.537**-9./375

Table 3: Results of Thin Layer Chromatographic fingerprinting

Extract	Mobile Phase	Number of spots*	R_f
Ethanol (hot extraction)	Ethyl-Acetate/Hexane 9; 1	2	0.3 0.9
	Ethyl-Acetate/Methanol	4	0.5 0.6 0.7 0.8
Water (hot extraction)	Ethyl –Acetate/n-Hexane 9; 1	-	-
	Ethyl-Acetate/Methanol 9; 1	2	0.2 0.4
Ethanol (cold extraction)	Ethyl-Acetate/n-Hexane 9; 1	2	0.3 0.9
	Ethyl-Acetate/Methanol	4	0.4 0.5 0.6 0.7
Water (cold extraction)	Ethyl-Acetate/n-Hexane 9;1	-	-
	Ethyl-Acetate/Methanol 9;1	2	0.2 0.4

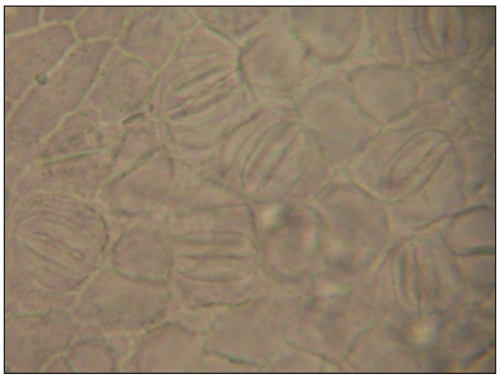
*Spots were visualized by spraying with 10% H_2SO_4 followed by heating at 105°C for 5 minute

Table 4: Fluorescence analysis of the powder.

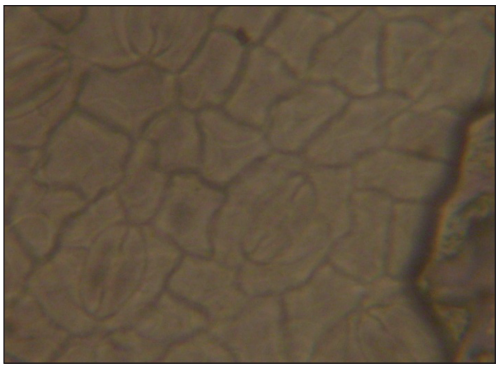
Solvent used	Daylight	UV Light	
		254 nm	366
Distilled water	No color change	Brown	Dark Green
Methanol	Brownish green	Brownish green	Dark green
Ethyl-acetate	No color change	Green	Dark green
Dichloromethane	Green	Dark green	Dark green
$FeCl_3$	Yellowish green	Yellowish green	Green
10% H_2SO_4	No Color change	No color change	Dark green

DISCUSSION

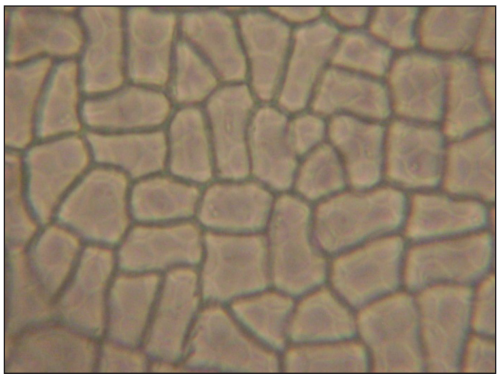
A transverse section of the leaves reveals the presence of a single layered epidermal cell on both surfaces covered with cuticle as well as trchomes. The mesopyll is diffentiated into palisade and spongy mesphyll. The palisade mesophyll is two layered tightly packed long cylindrical cells while the spongy mesophyll is single layer with intercellular spaces in between the cells. The vascular bundles is located between the mesophyll



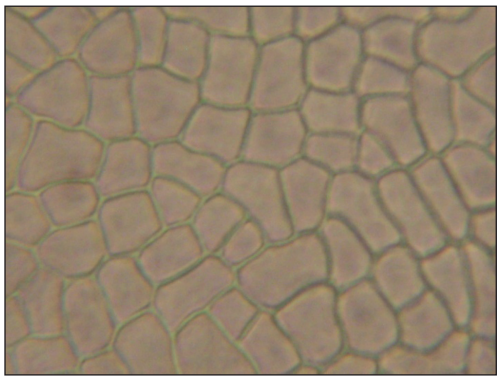
Abaxial epidermis with paracytic stomata



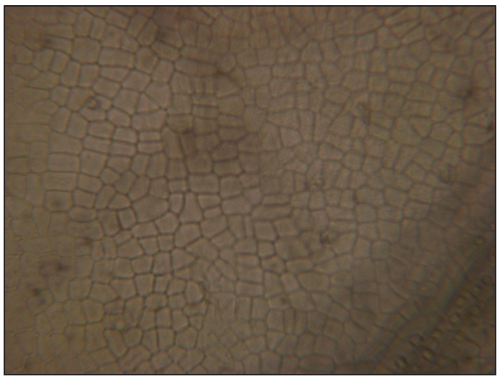
Abaxial surface showing the presence of paracytic stomata



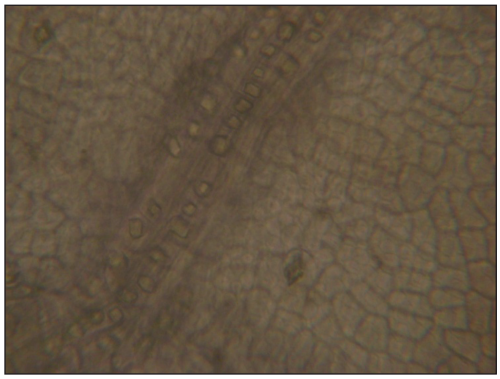
Adaxial epidermal cells



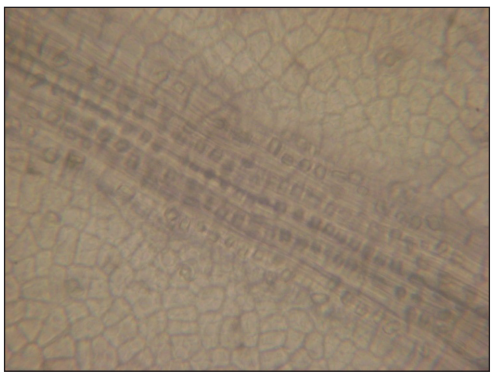
Adaxial epidermal cells under high power



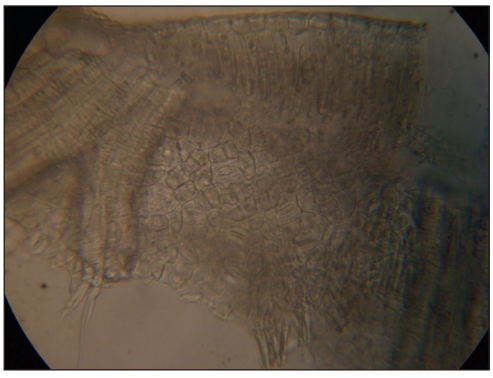
Adaxial surface of A altissimum under low power



Adaxial surface showing the presence of calcium oxalate crystals deposited along the veins under low power

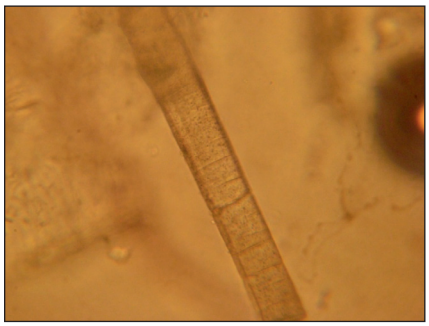


Adaxial surface showing the presence of Calcium oxalate crystals under low power



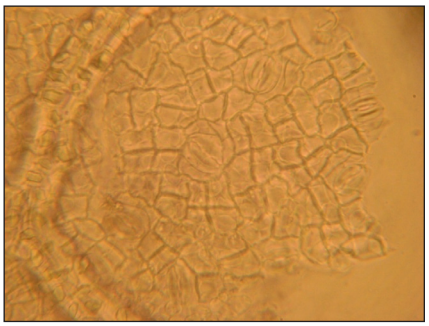
Transverse section of A altissimum under low power

Plate 1: Microscopical characteristics of the leaf of *A. altissimum*



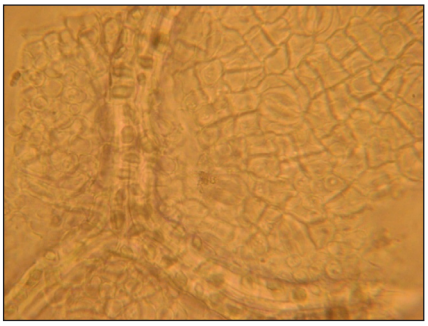
Fragments of powdered leaf showing fibre under high power

Fragments of powdered leaf showing fibre under low power

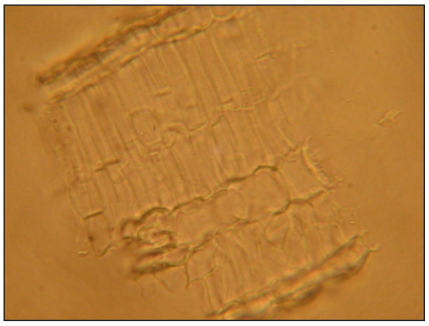


Fragments of powdered leaf showing calcium oxalate crystals, paracytic stomata and epidermal cells

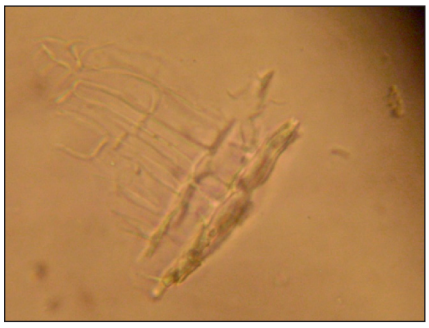
Powdered microscopy showing the presence of fibre



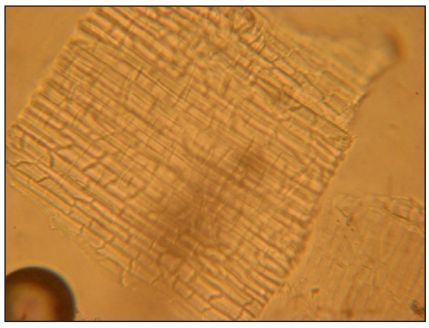
Fragments of powdered leaf showing calcium oxalate crystals, epidermal cell and paracytic stomata



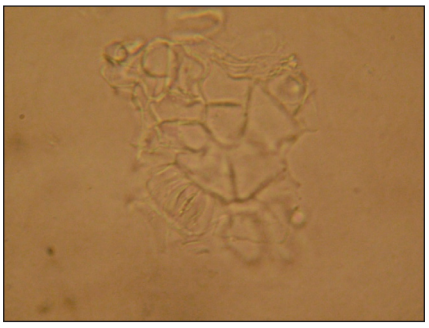
Powdered microscopy showing fragments of Epidermal cells, Palisade mesophlly and Spongy mesophyll and Lower epidermis and cuticle



Fragments of powdered leaf showing epidermal cells, and palisade mesophyll



Fragments of group of fibre cells



Fragments of epidermal cells and stomata

Plate 2: Powdered microscopy of *A. altissimum*

Stomata are present only on the lower surface and the stomata type is paracytic

Druse calcium oxalate crystals are present and are located along the veins

The powdered microscopy shows the presence of fragments of epidermal cells, palisade cells and spongy mesophyll as well as crystals of druse calcium oxalate crystals on the surfaces. The physical constant evaluation of the drugs is an important parameter in detecting adulteration. The ash values, extractive values and moisture content of stems were determined.[13] Ash values of a drug give an idea of the earthy matter or the inorganic composition and other impurities present along with the drug Extractive values are primarily useful for the determination of exhausted or adulterated drugs.[14]

CONCULSION

The parameters obtained in this study can be use as a basis for the establishment of monograph for the plant.

ACKNOWLEDGEMENT

Assistance of Ajibesin KK and Raji R.

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Pharmacognostic studies on the leaves of *Manilkara zapota* L. (*Sapotaceae*)

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ABSTRACT

Manilkara zapota L. (Sapotaceae) leaves have been reported to exhibit antibacterial and antioxidant activities. Establishment of pharmacognostic profile of the leaves will assist in standardization for quality, purity and sample identification. The present study was carried out to investigate macroscopical, microscopical and physiochemical parameters of *Manilkara zapota* L. leaves. Some of the diagnostic features of the leaves are the presence of trichomes, calcium oxalate prism, vessels, stomata, epidermis, and collenchyma cells. All the parameters were studied according to WHO guidelines. The determination of these characters will help future researchers in phytochemical as well as pharmacological analysis of this species.

Key words: *Manilkara zapota* L., Leaves, Pharmacognostic

INTRODUCTION

Pharmacognosy is defined as the study of physical, chemical, biochemical and biological properties of drugs[1]. Pharmacognostical studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy[2]. Research in pharmacognosy includes phytochemistry, microbial chemistry, biosynthesis, biotransformations, chemotaxonomy and other biological and chemical sciences.

Manilkara zapota L. belongs to the family *Sapotaceae*. It is an evergreen, glabrous tree, 8-15 m in height. It is cultivated throughout India, though it is native to Mexico and Central America. The seeds are aperients, diuretic tonic and febrifuge. Bark is antibiotic, astringent and febrifuge. Chicle from bark is used in dental surgery. Fruits are edible, sweet with rich fine flavour. Bark is used as tonic and the decoction is given in diarrhoea and peludism[3]. The leaves are used to treat cough, cold, and diarrhoea[4,5]. Bark is used to treat diarrhoea and dysentery[4]. Antimicrobial and antioxidant activities are also reported from the leaves[6,7,8].

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The objective of the present study was to evaluate various pharmacognostic standards like macroscopy and microscopy study of leaves; total ash, loss on drying, acid insoluble ash, water soluble ash, extractive values and microscopic characteristics of *Manilkara zapota* L. leaves powder.

MATERIAL AND METHODS

Plant material

Fresh leaves of *Manilkara zapota* L. were collected in the month of August, 2009, from Jam-Jodhpur, Jamnagar, Gujarat, India. The plant was compared with voucher specimen (voucher specimen No. PSN429) deposited at Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The leaves were separated, washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in air tight bottles.

Pharmacognostic studies

Macroscopy study

The plant was macroscopically examined for shape, size, surface characteristics, texture, color, consistency, odour, taste, etc[9].

Microscopy study

Microscopic studies were done by preparing a thin hand section of midrib and lamina region of *Manilkara zapota* L. leaf. The section was stained by saffranin. The powder

of the dried leaf was used for the observation of powder microscopic characters[9].

Physicochemical parameters

Physicochemical parameters like total ash value, loss on drying, water soluble ash, acid insoluble ash value, petroleum ether soluble extractive, acetone soluble extractive, methanol soluble extractive and water soluble extractive values were determined as per WHO guideline (2002)[10].

RESULTS AND DISCUSSION

Pharmacognosy study

The pharmacognostical study is the major and reliable criteria for identification of plant drugs. The pharmacognostic parameters are necessary for confirmation of the identity and determination of quality and purity of the crude drug. The detailed and systematic pharmacognostical evaluation would give valuable information for the future studies. The plant *Manilkara zapota* L. showed general characteristics of a dicot plant.

Macroscopically the leaf was green in colour. Apex and base were acute, margin was entire leathery, shape was oblong and petioles were 4.5 cm long (Fig. 1). The microscopy study revealed the presence of lower and upper epidermis, xylem, phloem, vascular bundles, mesophyll, trichome and collenchyma. Mesophyll was differentiated into palisade and spongy parenchyma. Palisade was formed from compactly arranged elongated, narrow columnar cells with beaded anticlinal walls. Palisade cells were filled with chlorophyll. Spongy parenchyma was made up of parenchymatous cells with varying size and shape. Vascular bundles were arc shaped. Xylem was lignified and phloem was non-lignified. Unicellular trichome was observed on the epidermis (Fig. 2).

Microscopy study of powder showed the presence of upper epidermis cells which were thick and irregular walled, prisms of calcium oxalate crystals, simple unicellular trichome, simple pitted vessels, actinocytic stomata and xylem vessels in longitudinal sectional view showed spiral thickening (Fig. 3).

Physicochemical characters

The moisture content of dry powder of leaves of *Manilkara zapota* L. was 8.0 % which seems to be lower than that necessary to support the growth of microbes to bring any change in the composition of the drugs. Physical constant like ash value of the drug gives an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. Extractive values are useful for the determination of exhausted or

Figure 1: Macroscopic characteristics of Manilkara Zapota L. leaf

adulterated drugs. The results of the physical constants of the drug powder are given in Table 1. Total ash of crude powder of leaves of *Manilkara zapota* L. was 6.15 %, acid insoluble ash was 0.85 % and water soluble ash was 7.6 %. Low amount of total ash, acid insoluble ash and water soluble ash indicate that the inorganic matter and non physiological matter such as silica is less in leaves

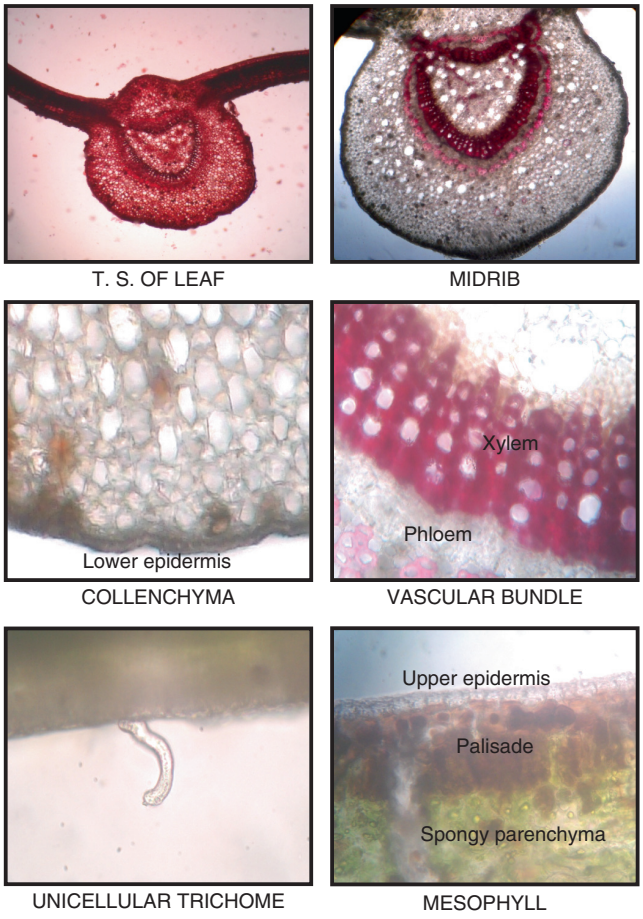

Figure 2: Photomicrographs of microscopic characteristics of Manilkara Zapota L. leaf

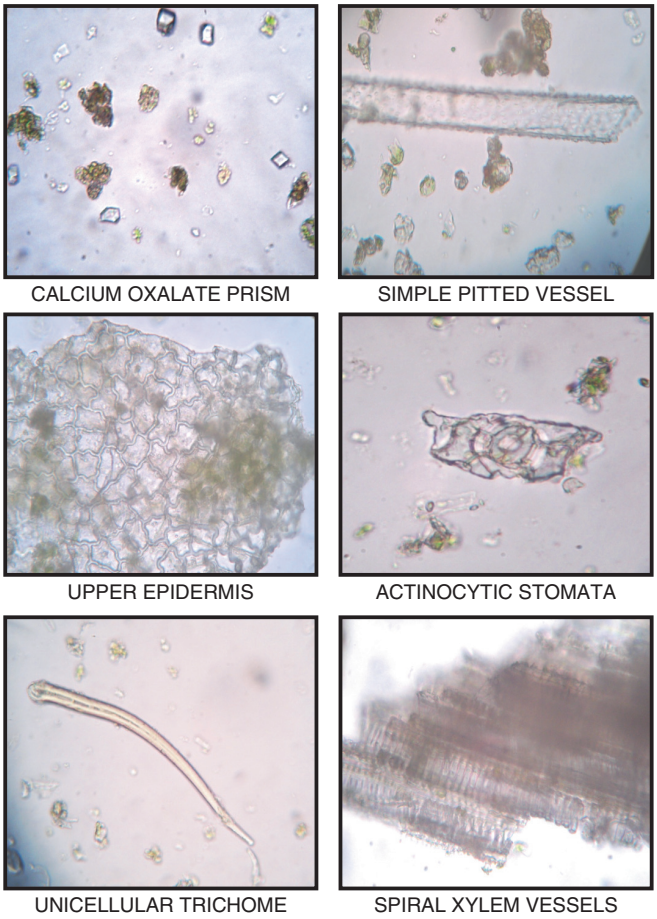

Figure 3: Photomicrographs of microscopic characteristics of powder of Manilkara Zapota L. leaf

Table 1: Physicochemical characterization of powder of leaves of *M. zapota*

Parameters	Yield % (w/w)
Loss on drying	8.00
Total ash	6.15
Acid insoluble ash	0.85
Water soluble ash	7.60
Petroleum ether soluble extractive	4.41
Acetone soluble extractive	15.66
Methanol soluble extractive	4.97
Water soluble extractive	18.23

of *Manilkara zapota* L. Extractive yield of leaves of *Manilkara zapota* L. was highest in water (18.23 %), followed by acetone (15.66 %) and methanol (7.97 %). Lowest yield was in petroleum ether (4.41 %). The variation in extractable matter in various solvents is suggestive of the fact the formation of the bioactive principle of the medicinal plant is influenced by number of intrinsic and extrinsic factors. High water soluble and alcohol soluble extractive value reveal the presence of polar substance like phenols, tannins and glycosides, as also reported by Sharma et al., (2009)[11].

Manilkara zapota L. is used for the treatment of various diseases therefore it is important to standardize it for use as a drug. The pharmacognostic constants for the leaves of this plant, the diagnostic microscopic features and the numerical standards reported in this work could be useful for the compilation of a suitable monograph for its proper identification. Pharmacognostic studies on different plants like *Polyalthia longifolia* var. pendula Leaf[12], *Manilkara hexandra* (Roxb.) Dubard leaf[13], *Punica granatum* L. leaf[14], *Cissus quadrangularis* L. stem[15], *Psidium guajava* L. leaf[16] has been reported.

CONCLUSION

Standardization of herbal drugs is very much essential as they are derived from heterogeneous sources which can lead to variations. These variations can lead to erroneous results in various pharmacological and phytochemical studies. *Manilkara zapota* L. leaves are known for many medicinal properties hence the present study may be useful to supplement information in respect to its identification, authentication and standardization; no such data is available for the same till date.

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Studies on Medicinal Plant Resources of the Himalayas: GC-MS Analysis of Seed Fat of Chyuri (*Diploknema butyracea*) from Nepal

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ABSTRACT

Chyuri (*Diploknema butyracea* (Roxburgh) H. J. Lam) is widely used in Nepal for its medicinal properties and as a source of Chyuri ghee (fat) extracted from the seeds. The main purpose of the present study was the GC-MS analysis of fatty acids in seed fat of Chyuri after acidic methanolysis. Palmitic acid methyl ester (**1**), linoleic acid methyl ester (**2**), oleic acid methyl ester (**3**) and steric acid methyl ester (**4**) were identified as the main components. Ethnomedicinal use and distribution of Chyuri in Nepal is also discussed.

Key words: Chyuri, *Diploknema butyracea,* GC-MS, Nepal

INTRODUCTION

Chyuri (*Diploknema butyracea* (Roxburgh) H. J. Lam; *Bassia butyracea* Roxburgh; *Madhuca butyracea* (Roxburgh) Macbride; *Aesandra butyracea* (Roxburgh) Baehni), belonging to family Sapotaceae is a deciduous tree about 20 m high. It is distributed throughout Nepal mainly in the sub-Himalayan tracts on open hillsides of 300 to 1,500 meters, and also in northern India and Bhutan.[2] It is also named as Indian butter tree and the main product of the tree is ghee or fat, extracted from the seeds and named as Chyuri ghee or Phulwara butter.

Various parts of Chyuri plant are used by different ethnic groups of Nepal for medicinal and other purposes. Seed fat is applied for headache, rheumatism, boils and pimples. It is also used as emollient for chapped hands and feet in winter. Juice of the bark is used for the treatment of indigestion, asthma, rheumatism and boils and also as anthelmintic. Juicy pulp of ripe fruit is eaten fresh. Juice of the corolla is boiled into a syrupy liquid, which villagers use like syrupy sugar.[2,3] Chyuri tree is socially, culturally and economically important to the *Chepangs,* an indigenous ethnic group of people living in dense forests of mid-Southern Chitwan district of Nepal, who call it *Yoshi* in their language. Fruit makes a dietary supplement for *Chepang* people. The fat extracted from seed is used for cooking and lighting lamps. Some people use it as hair oil and raw material for soap. Bark and oil cake are used as fish poison. Oil cake is also used as fertilizer to protect crops from harmful insects and worms.[4]

Previous phytochemical studies have isolated butyraceol,[5] MI-saponin A, 16α-hydroxy MI-saponin A, butyrosides A, B,[6] C, D,[7] from the seeds of *D. butyracea*. Similarly, butyracic acid and myricetin-3-*O*-rhamnoside were isolated from the leaves.[8] α-Spinasterol, β-sitosterol glucoside, α-amyrin acetate, β-amyrin acetate and 3β-palmitoxy-olea-12en-28-ol were isolated from bark and fruit pulp.[9] Flavonoids, quercetin and dihydro-quercetin were isolated from the nut-shell[10] and quercetin, quercetin-3-*O*-rhamnoside, myricetin and myricetin-3-*O*-rhamnoside from the flowers.[11] Various studies have reported the triglyceride and fatty acid composition of Phulwara butter collected from India[12-14] but no such studies are reported for the analysis of Chyuri ghee from Nepalese sources. Hence, present investigation was carried out to study the components in seed fat and distribution of Chyuri plant in Nepal.

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EXPERIMENTAL

Plant material and extraction of seed oil

Chyuri seeds used in this study were collected from a habitat of Nepalgunj, Nepal by the authors on the three times of expeditions in 2007 (Sample A), 2008 (Sample B) and 2009 (Sample C). The seed oil was extracted by the traditional method.[4] The ripe Chyuri fruits were squeezed to remove their mesocarp. The seeds were then separated, cleaned, boiled, dried and then crushed to obtain seed flour. The flour was then steamed in a perforated bamboo basket and placed over a boiling metal pot for steaming. The steamed flour was then squeezed to extract the fat.

Sample preparation

Ten mg of each sample were subjected to methanolysis by refluxing with 1ml of freshly prepared 5% HCl-MeOH at 70 ºC for 6 hours. The solvent was evaporated, and these samples were dissolved in hexane (1 ml) and subjected to the gas chromatography.

Seed fat analysis

The gas chromatographic analysis was performed on a Hewlett Packard Series II 5890 under the following conditions: Equity™-5 Fused silica capillary column (30 m × 0.25 mm × 0.25 m); column temperature 150 ºC (initial) to 250 ºC at 5 ºC/min; carrier gas He; flow rate 1.22 ml/min; volume injected 1 l dissolved in hexane. Quantification of relative amount of each component was done according to the area percentage method. Gas chromatography-mass spectrometry (GC-MS) analysis was performed in a JEOL MS route (JMS 600W) mass spectrometer, operating at 70 v. Identification of individual components was based on the comparision of mass spectra with NIST/EPA/NIH Mass Spectral Library.

Distribution data

To study the distribution pattern of Chyuri, voucher specimens deposited in the National Herbarium and Plant Laboratories, Godawari, Kathmandu, Nepal were investigated. Reported locality, altitude and GPS data for the specimens studied are given in Table 2.

RESULTS AND DISCUSSION

Chyuri plant has medicinal and economical importance to various ethnic communities in Nepal. Chyuri seed fat is one of the main components used both as medicine for various ailments and commercial source for pharmaceutical, cosmetic and soap industries. The results of GC-MS analysis after methanolysis of seed fat showed the presence of palmitic acid methyl ester (**1**), linoleic acid methyl ester (**2**), oleic acid methyl ester (**3**) and steric acid methyl ester (**4**) in all three samples A, B and C at almost similar composition (Table 1). Representative gas chromatogram of sample B is given in Figure 1. Palmitic acid methyl ester (**1**) was the main component in all three samples followed by oleic acid methyl ester (**3**). The relative composition of seed fat was found to be similar with the reported data for from previous studies collected from India.[12-14] These studies suggested that the Chyuri ghee has high potential to be used in the manufacture of, cosmetics, soaps and in pharmaceutical and confectionery industry. The fatty acid composition is also important from the point that commercial ghee in Nepal is often adulterated with Chyuri seed oil.[15]

Study of the voucher specimens deposited in the National Herbarium and Plant Laboratories, Godawari, Kathmandu, Nepal has revealed that it is widely distributed from eastern part to western part of Nepal at altitude from 150 m to 1620 m (Table 2.) The proper cultivation and utilization of Chyuri tree as a medicinal species and source of seed fat may improve the social and economic status of Nepalese people. Further bio-assay guided chemical analysis of the different plant parts of Chyuri may help in the development of evidence based medicines. Similarly, study on the altitude variation of components in seed oil would be interesting for the selection of proper biological source.

Table 1: Relative composition of Chyuri seed fat in samples A, B and C[a]

Component	Sample A	Sample B	Sample C
palmitic acid methyl ester (**1**)	66.0%	58.5%	62.1%
linoleic acid methyl ester (**2**)	2.6%	3.4%	3.4%
oleic acid methyl ester (**3**)	29.0%	35.2%	30.6%
steric acid methyl ester (**4**)	2.4%	2.9%	3.9%

[a]percentages were calculated from the peak area.

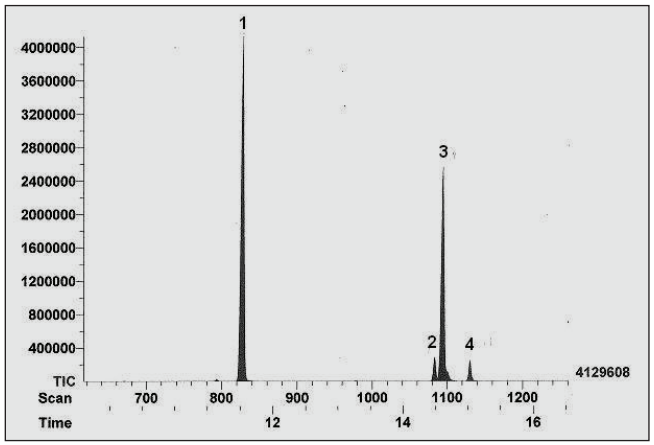

Figure 1: Gas chromatogram of Chyuri seed fat (Sample B).

Table 2: Distribution pattern of Chyuri tree in Nepal

S.N.	District	Locality	Altitude	Longitude (E)	Latitude (N)
1	Baglung	Raigha	850 m	83°02'	28°02'
2	Baitadi	Khateda - Patan	1200 m	80°03'	29°03'
3	Bajhang	Chin	1100 m	81°01'	29°04'
4	Banke	Khajura	180 m	83°04'	27°04'
5	Banke	Nepalgunj	150 m	81°37'	28°03'
6	Chitwan	Tungati	380 m	85°59'	27°42'
7	Chitwan	Lothar	150 m	84°45'	27°42'
8	Dadeldhura	Kalina - Gogan	1000 m	80°02'	29°02'
9	Dang	Ramche	1395 m	82°02'	28°01'
10	Dang	Dharampani	900 m	82°16'	28°00'
11	Dhankuta	Dharapani	1200 m	86°41'	27°18'
12	Jajarkot	Jhapra	1500 m	82°01'	28°05'
13	Kabhre	Amaltari	680 m	86°08'	27°38'
14	Kabhre	Mukpa	1460 m	83°40'	28°31'
15	Kaski	Pokhara	800 m	83°59'	28°13'
16	Makawanpur	Champ kharka	1450 m	85°01'	27°03'
17	Makwanpur	Kamle	1000 m	85°04'	27°01'
18	Myagdi	Myagdi	1080 m	83°31'	28°20'
19	Pyuthan	Bijuwar	780 m	82°01'	28°01'
20	Ramechap	Hulak	1000 m	86°16'	27°44'
21	Rukum	Banphikot	1000 m	82°02'	28°04'
22	Salyan	Cherneta	1620 m	83°36'	28°19'
23	Sindhupalchok	Barabise	500 m	85°04'	27°05'
24	Sunsari	Barahchhetra	170 m	87°02'	26°05'
25	Surkhet	Babai	180 m	81°02'	28°03'
26	Surkhet	Kalyan	870 m	81°03'	28°04'
27	Udaypur	Trijuga	185 m	86°03'	26°05'

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TLC Detection of β-sitosterol in *Michelia champaca* L. Leaves and Stem Bark and it's Determination by HPTLC

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ABSTRACT

Introduction: *Michelia champaca* L. (Magnoliaceae) is an ancient Indian medicinal plant a native of the Indian subcontinent and possessing numerous traditional uses. β-sitosterol is an important plant sterol present in Michelia which is reported to posses' chemopreventive and adaptogenic properties. In the present study, High Performance Thin Layer Chromatography has been developed for detection, and quantification of β-sitosterol in *Michelia champaca* (leaves and stem-bark), after its detection and characterization initially by TLC. **Methods:** Increasing serial dilutions of reference standard β-sitosterol (200 to 1000 µg mL^{-1}) were scanned at 273 nm to detect and quantify the concentrations of β-sitosterol in the test samples. **Results:** The estimated values obtained from the same were 791.726 µg mL^{-1} and 696.446 µg mL^{-1} for leaves and stem bark respectively. Leaves were found to be the richest source of β-sitosterol in *Michelia champaca*. **Conclusion:** The method provided a rapid and easy approach for detection and the quantitation of the bio-marker β-sitosterol. The authors also aim to validate the present method in terms of ruggedness and accuracy and undertake the isolation of β-sitosterol from the said plant.

Key words: *Michelia champaca*, β-sitosterol, HPTLC, quantitation, TLC

INTRODUCTION

Michelia champaca L. (Magnoliaceae), commonly known as champa a native of Southern parts of India is cultivated in various parts of India and planted in gardens and near temples.[1-2] The glorious medicinal plant is a reservoir of numerous active principles and secondary metabolites and is extremely rich in its chemistry and is often widely used traditionally for indolent swellings, fevers and in nervousness.[3] Parthenolide from leaves and root bark, michampanolide, 8-acetoxyparthenolide magnograndiolide, costunolide, dihydroparthenolide and micheliolide from root bark and β-sitosterol, liriodenine, ushinsunine, magnoflorine from stem bark are some of the important chemical moieties reported from this plant.[4-10] β-sitosterol is reported to help in the management of ageing, hyperlipidaemia, cholesterol absorption, and as an immunomodulator. It is beneficial in the treatment of breast cancer and cancer of the prostate gland. It is also useful in certain gynecological disorders.[11-12] Recently it has been estimated in a number of herbal candidates like corn-silk,[13] *Rhizophora mucronata*[14] and *Soymida febrifuga*.[15] The structure of β-sitosterol is shown in Figure 1. Many methods like UV spectroscopy; HPLC, GC and HPTLC are available for determination of β-sitosterol in plants and plant products. In the present investigation, chromatographic fingerprint of *Michelia champaca* L., (leaves and stem-bark) has been developed by HPTLC method using β-sitosterol as a marker compound.

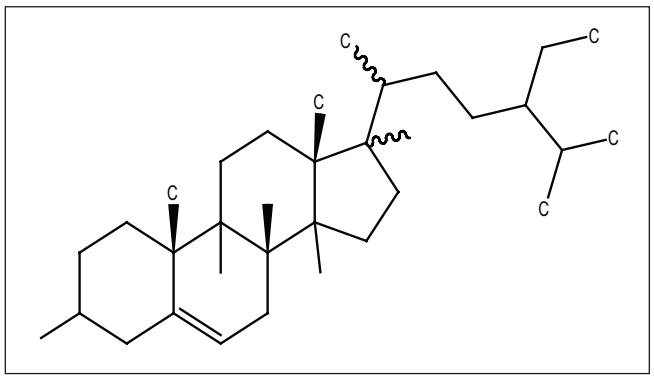

Figure 1: Structure of β-sitosterol

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MATERIALS AND METHODS

Plant Material

The plant material was collected in and around Lucknow, Uttar Pradesh in the month of August and authenticated by National Botanical Research Institute, Lucknow; also a voucher specimen was submitted for future reference (Ref No. NBRI/CIF/176/2010). The air dried plant material was size communited to a moderately fine powder (#355/180) and stored in an air-tight container for future/ further studies.

***Solvents*:** All the solvents used were of AR grade.

***Reference standard*:** The reference standard (β-sitosterol) was obtained from Sigma Aldrich, USA.

TLC Detection of β-sitosterol in Michelia champaca

β-sitosterol was detected in *Michelia champaca* L. leaves and stem-bark (methanolic extract) following permutations, combinations and optimization of solvent-systems. The results obtained are summarized in Table 1, on the basis of which solvent system for running HPTLC fingerprints was selected.

Table 1: Detection of β-sitosterol by TLC

S.No.	Solvent System	Detection	Rf	
			Leaves	Stem-bark
1	Toluene: Ethyl acetate (8:2)	Anisealdehyde-Sulphuric acid (stem) UV(leaves)	0.61	0.55
2	Benzene: Methanol (9:1)	Anisealdehyde-Sulphuric acid (stem) UV (leaves and stem-bark)	0.30	0.44
3	Toluene: Methanol (9:1)	Anisealdehyde-Sulphuric acid (stem) UV (leaves and stem-bark)	0.25	0.20

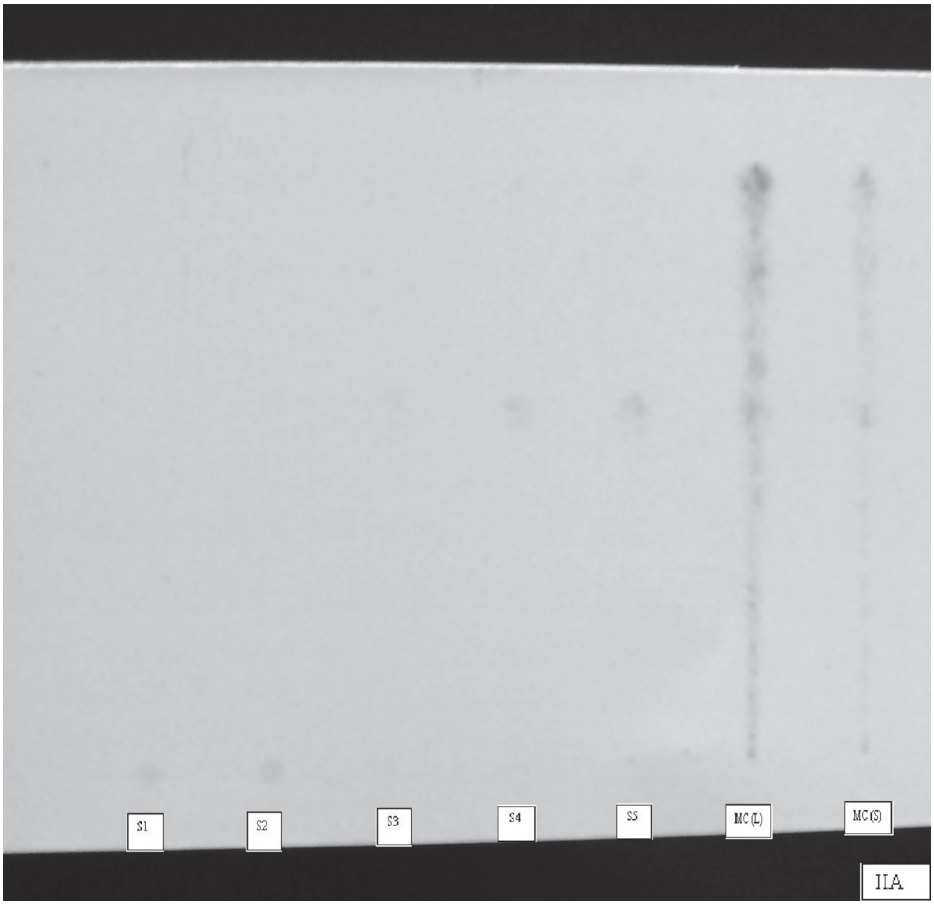

Figure 2: Chromatogram of β-sitosterol, (a) Detection: Anisealdehyde-Sulphuric acid

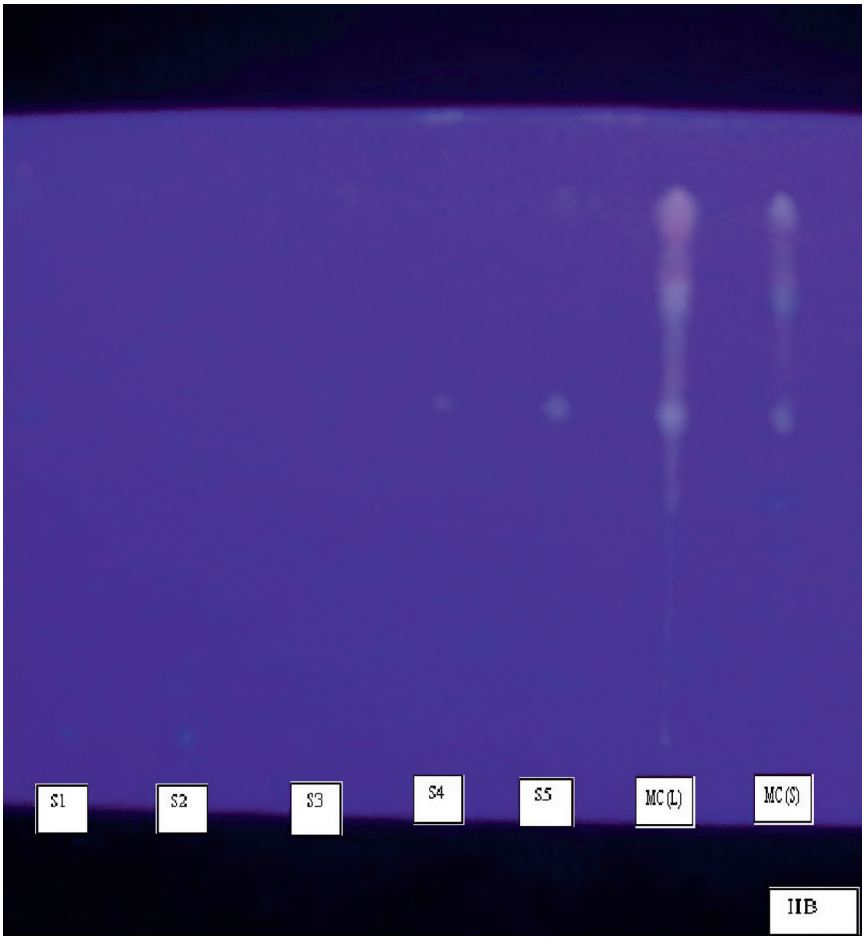

Figure 2: *Continued* (b) Detection: UV 254 nm
β-sitosterol working standards: S1:- 200 µg mL^{-1} S2:- 400 µg mL^{-1}, S3:- 600 µg mL^{-1}, S4:- 800 µg mL^{-1}, S5:- 1000 µg mL^{-1}

Chromatographic conditions for HPTLC

- **Instrument:** HPTLC system equipped with a sample applicator device Camag Linomat 5. Camag twin trough chamber, Camag TLC scanner and integration software (Wincats)
- **HPTLC Plate:** Silica gel GF254 (Merck) 15 × 10 cm
- **Mobile Phase:** Benzene: Methanol (9:1)[16]
- **Wavelength:** 273 nm

Standard Preparation

A stock solution of β-sitosterol (1000 µg mL^{-1}) was prepared by dissolving 10.0 mg of accurately weighed β-sitosterol in Methanol and diluting it to 10.0 mL with methanol.[17] Further dilutions were made with Methanol to obtain working standards 200, 400, 600, 800 and 1000 µg mL^{-1}.

Sample Preparation

100 mg of size reduced air dried powdered plant material (leaves, stem-bark) was Soxhlet extracted with methanol for 16 hours. The methanolic extract was concentrated and 10 mg of the concentrated methanolic extract was redissolved in 10 mL methanol to obtain a test sample (1000 µg mL^{-1})

Procedure

The TLC plate was activated by placing in an oven at the temperature of 110 °C for 20 min. the plate was spotted with test and standard preparation maintaining a distance of 15 mm from the edge of TLC plate. It was developed upto 75 mm in the twin trough chamber using mobile phase, dried in an oven and subjected for TLC scanning at 273 nm.[18-19]

RESULTS

The Rf's obtained with different solvent systems in TLC are enlisted in Table 1. Based on these results Benzene: Methanol (9:1) was selected for running HPTLC fingerprints, and the chromatograms are shown in Figure 2 (a-b). Under the chromatographic conditions described above, the Rf

value of β-sitosterol was about 0.50 and 0.51 in leaves and stem-bark of *Michelia champaca* respectively. The respective Rf's obtained for each track is shown in Table 2. The Chromatograms of standard β-sitosterol are shown in Figure 3 (a-e) and that of β-sitosterol in *Michelia champaca* are shown in Figure 4 (a-b). Spectral Comparison of β-sitosterol reference standard with β-sitosterol in samples is shown in Fig 5(a-d). The 3D spectra of all tracks scanned at 273 nm are shown in Figure 6 (a-c). The area under the curve (AUC)

Table 2: Rf range and maximum Rf (peak) of tracks 1-7

S.No.	Start position	Maximum Rf	End position
Track 1	0.46	0.56	0.58
Track 2	0.45	0.51	0.56
Track 3	0.41	0.53	0.59
Track 4	0.41	0.51	0.58
Track 5	0.42	0.51	0.57
Track 6	0.41	0.51	0.58
Track 7	0.41	0.50	0.58

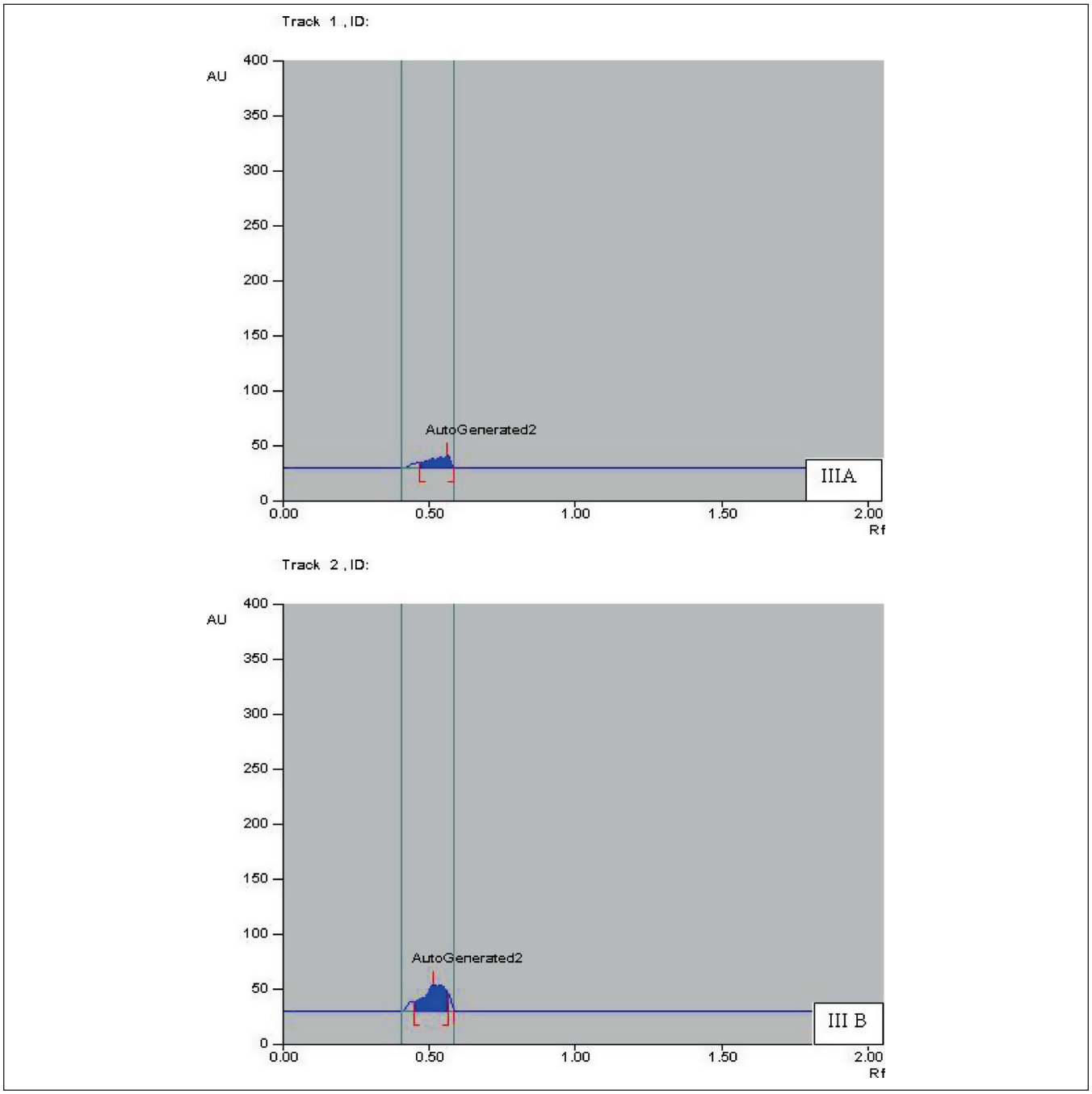

Figure 3: A Typical HPTLC chromatogram of β-sitosterol working standard (a) Track 1 (200 µg mL^{-1}) (b) Track 2 (400 µg mL^{-1})

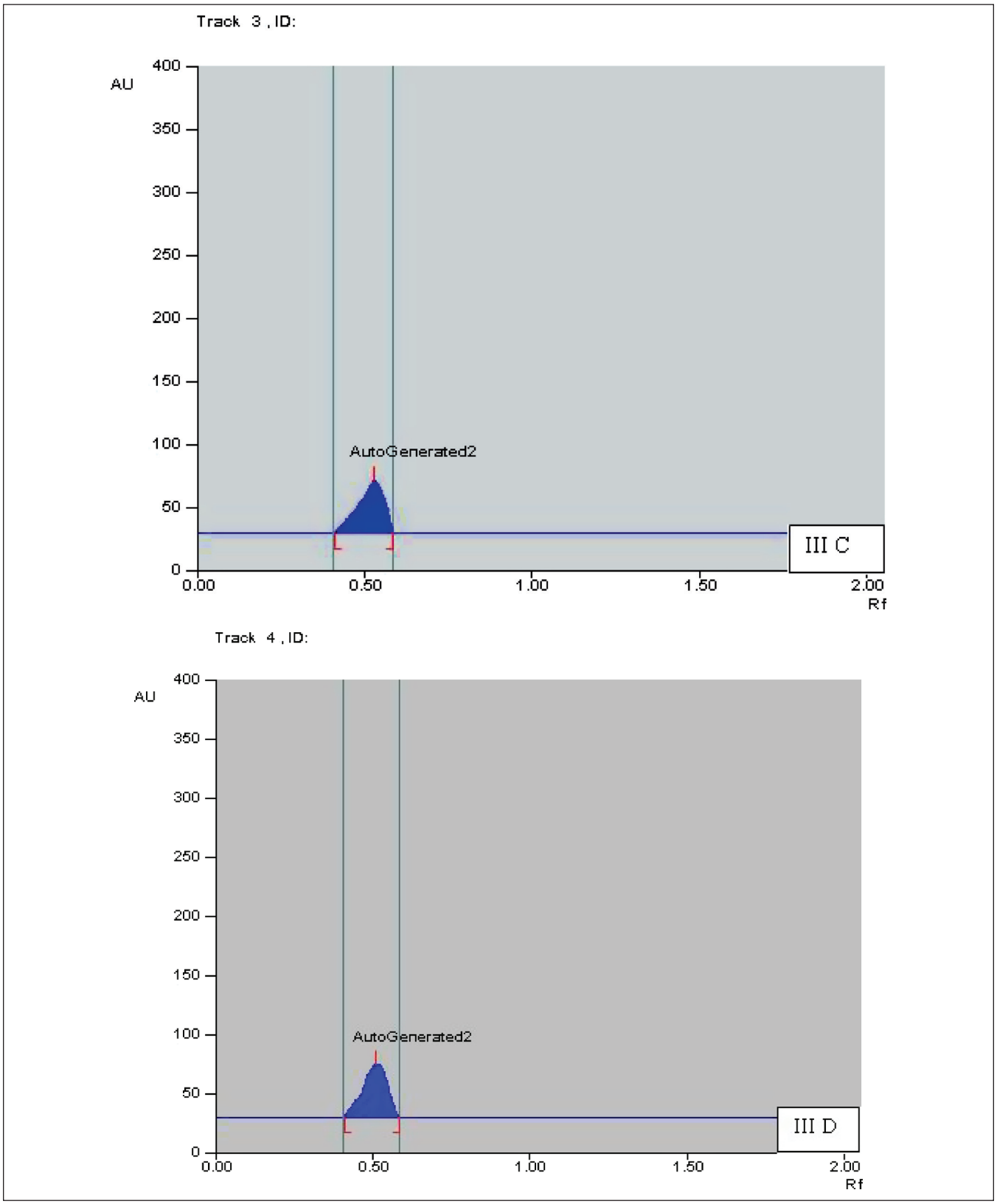

Figure 3: *Continued* (c) Track 3 (600 µg mL^{-1}) (d) Track 4 (800 µg mL^{-1})

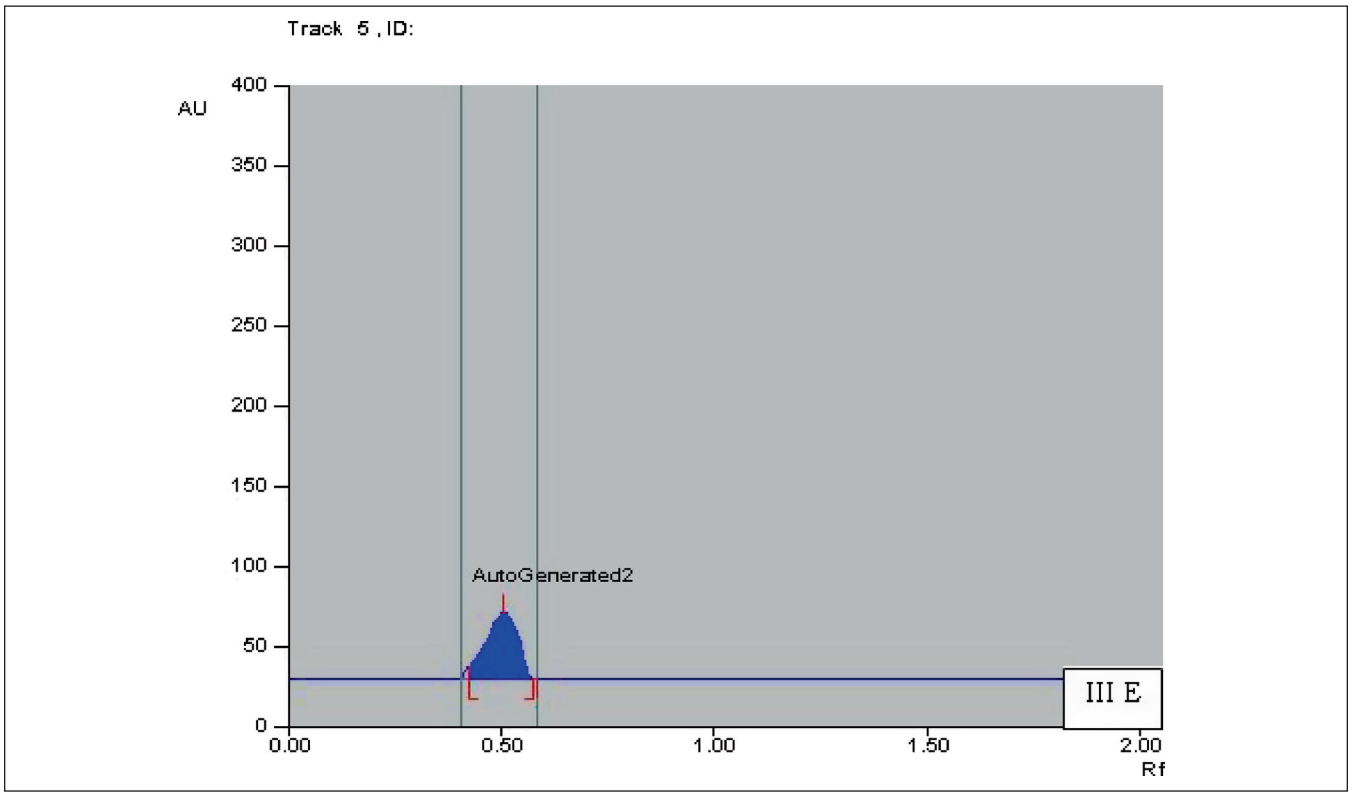

Figure 3: *Continued* (e) Track 5 (1000 µg mL^{-1})

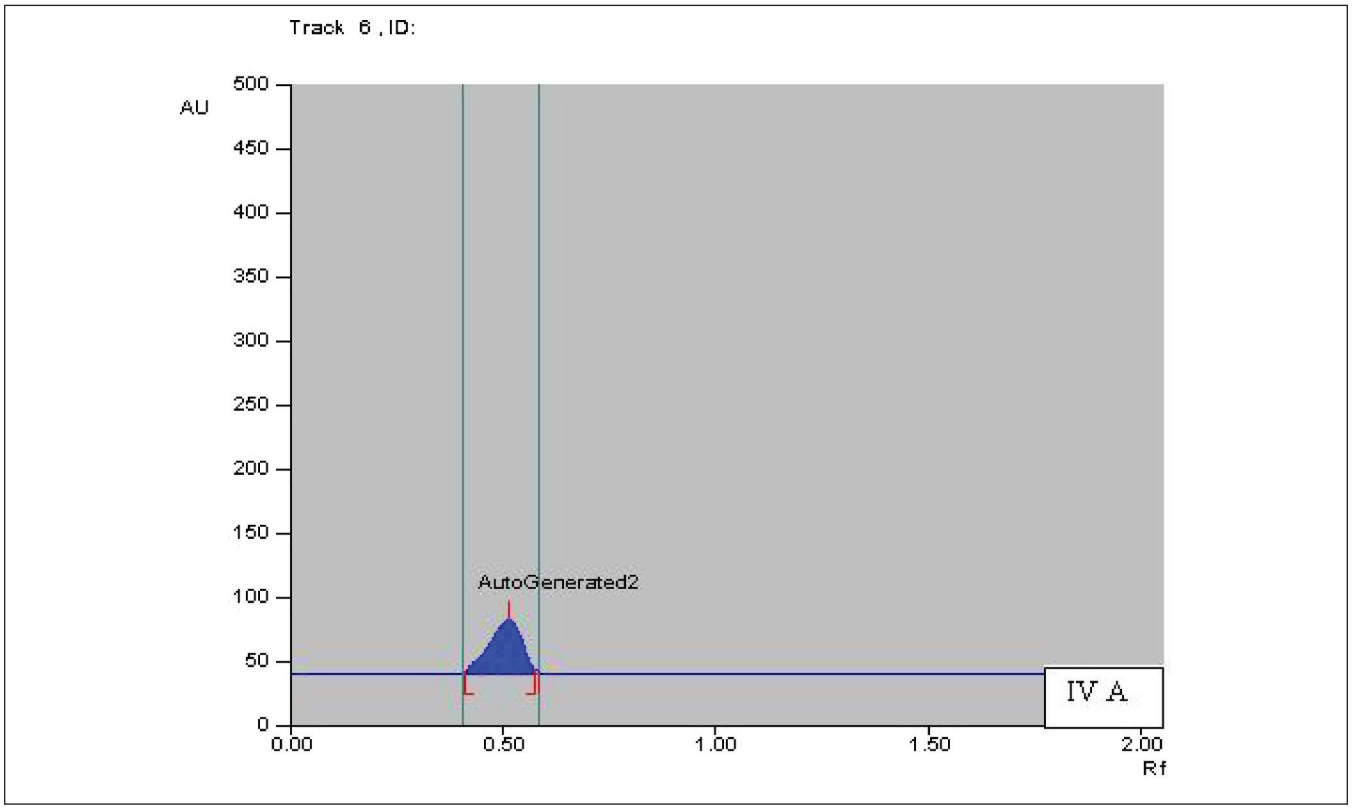

Figure 4: A Typical HPTLC chromatogram of β-sitosterol in *Michelia champaca* L. (a) Track 6 (leaves)

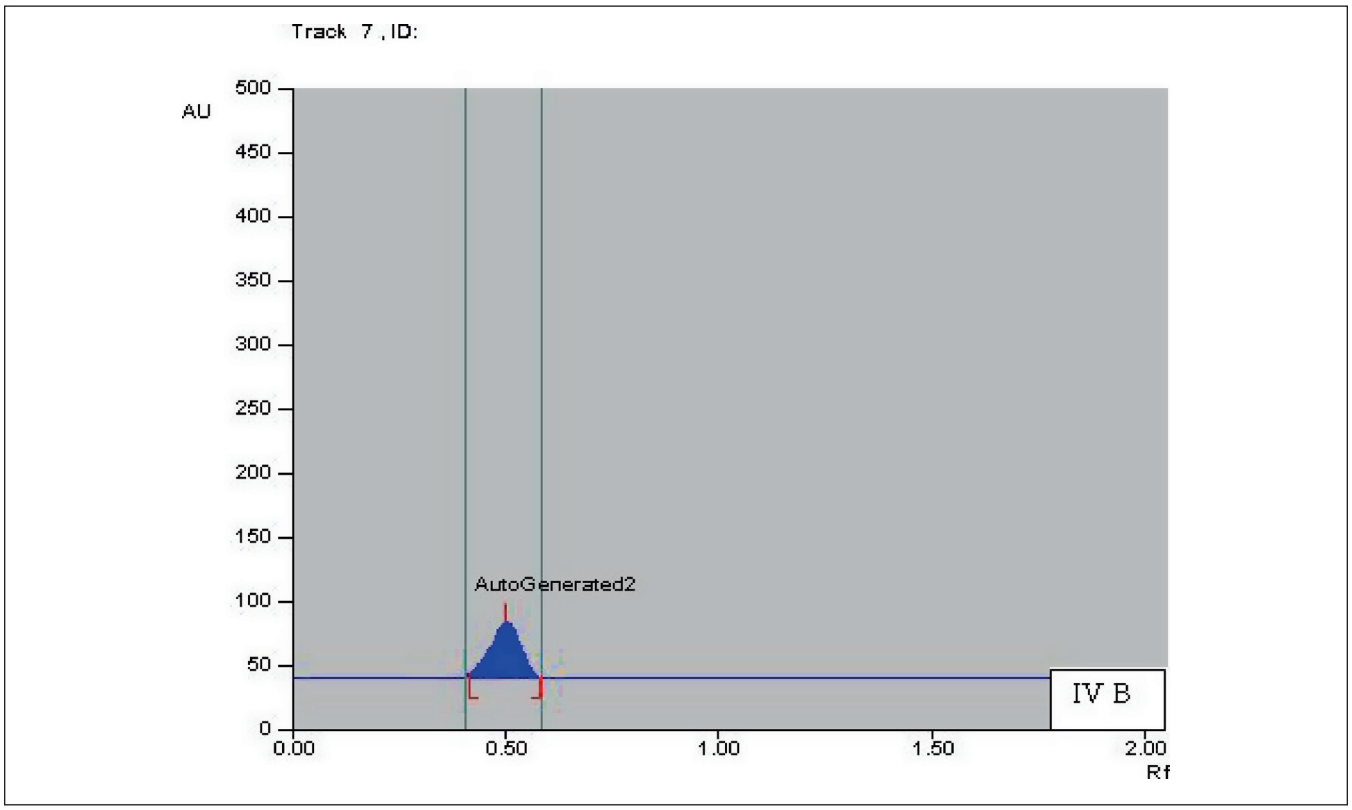

Figure 4: *Continued* (b) Track 7 (stem bark)

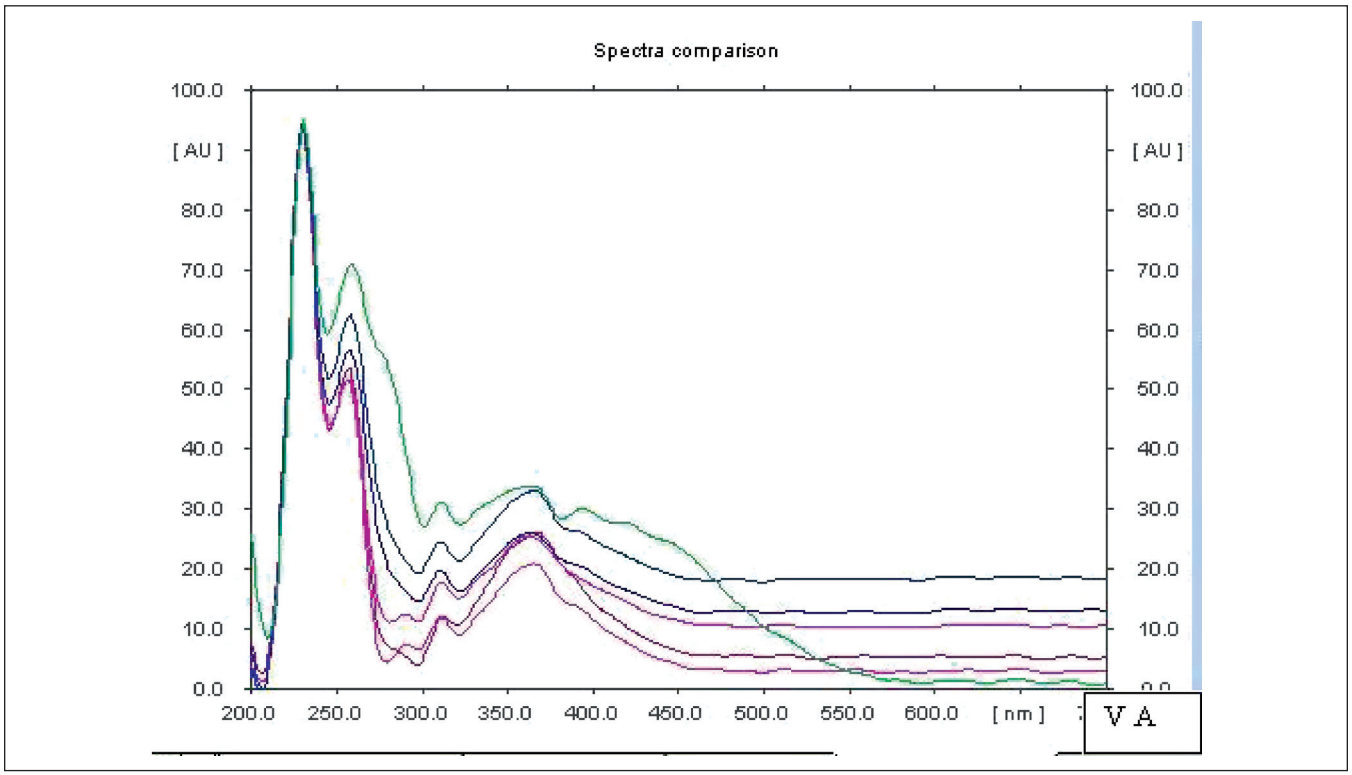

Figure 5: Spectral comparison of sample tracks with standards at selected wavelength. (a) Track 6 with Tracks (1-5) at 230 nm

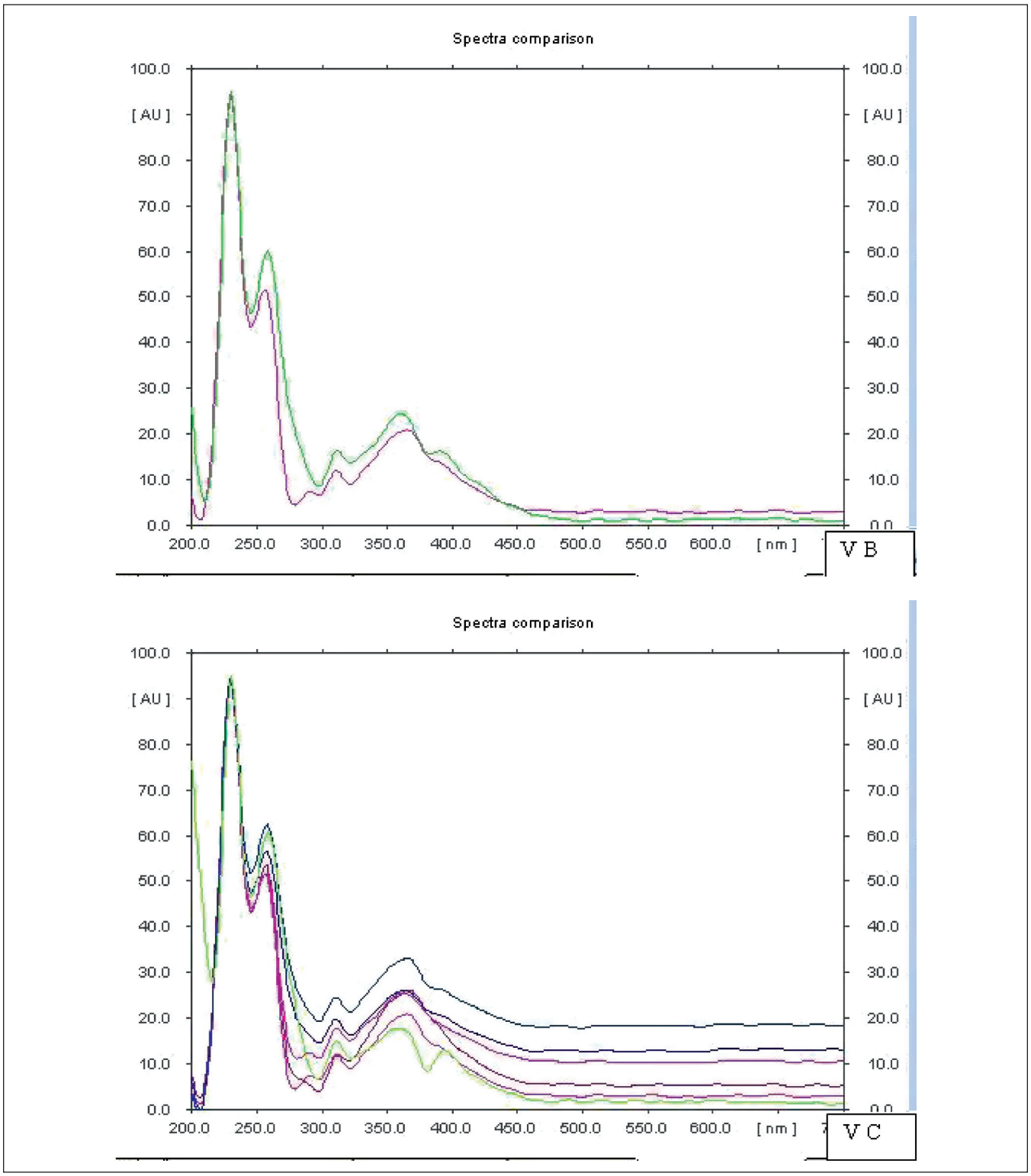

Figure 5: *Continued* (b) Track 6 with Track 2 at 230 nm (c) Track 7 with Tracks (1-5) at 230 nm

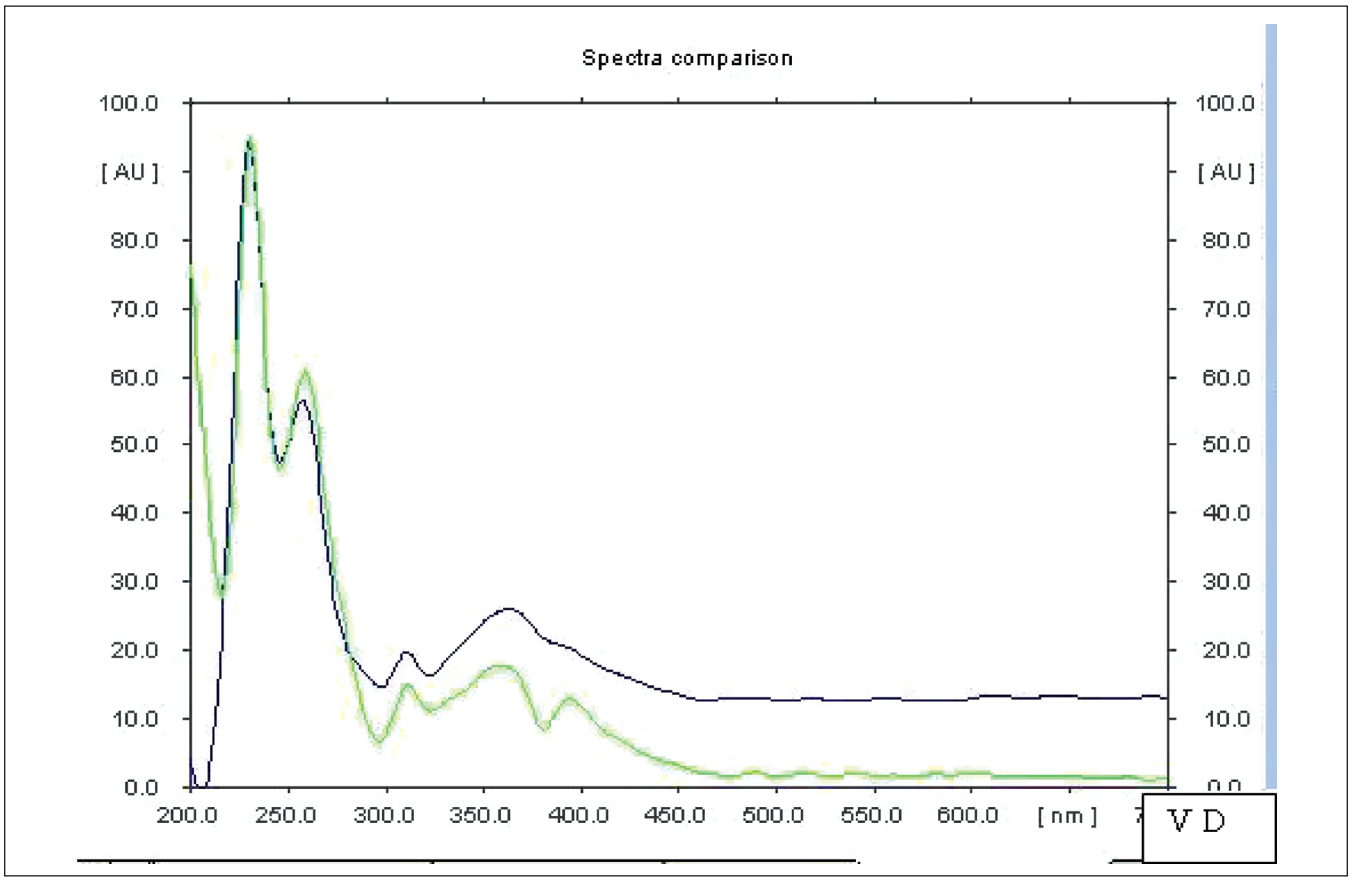

Figure 5: *Continued* (d) Track 7 with Track 4 at 230 nm

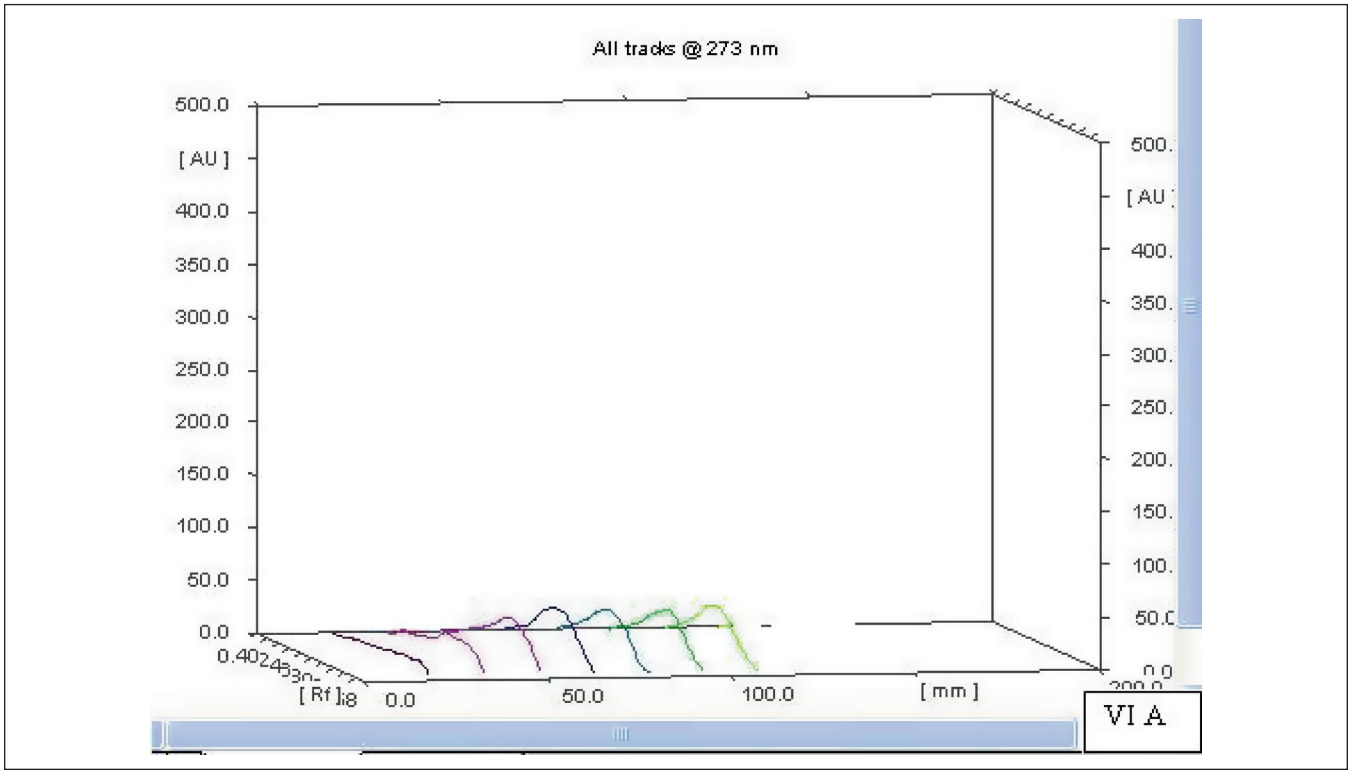

Figure 6: 3D spectra of Tracks 1-7 scanned at 273 nm at different vertices (a) 65°

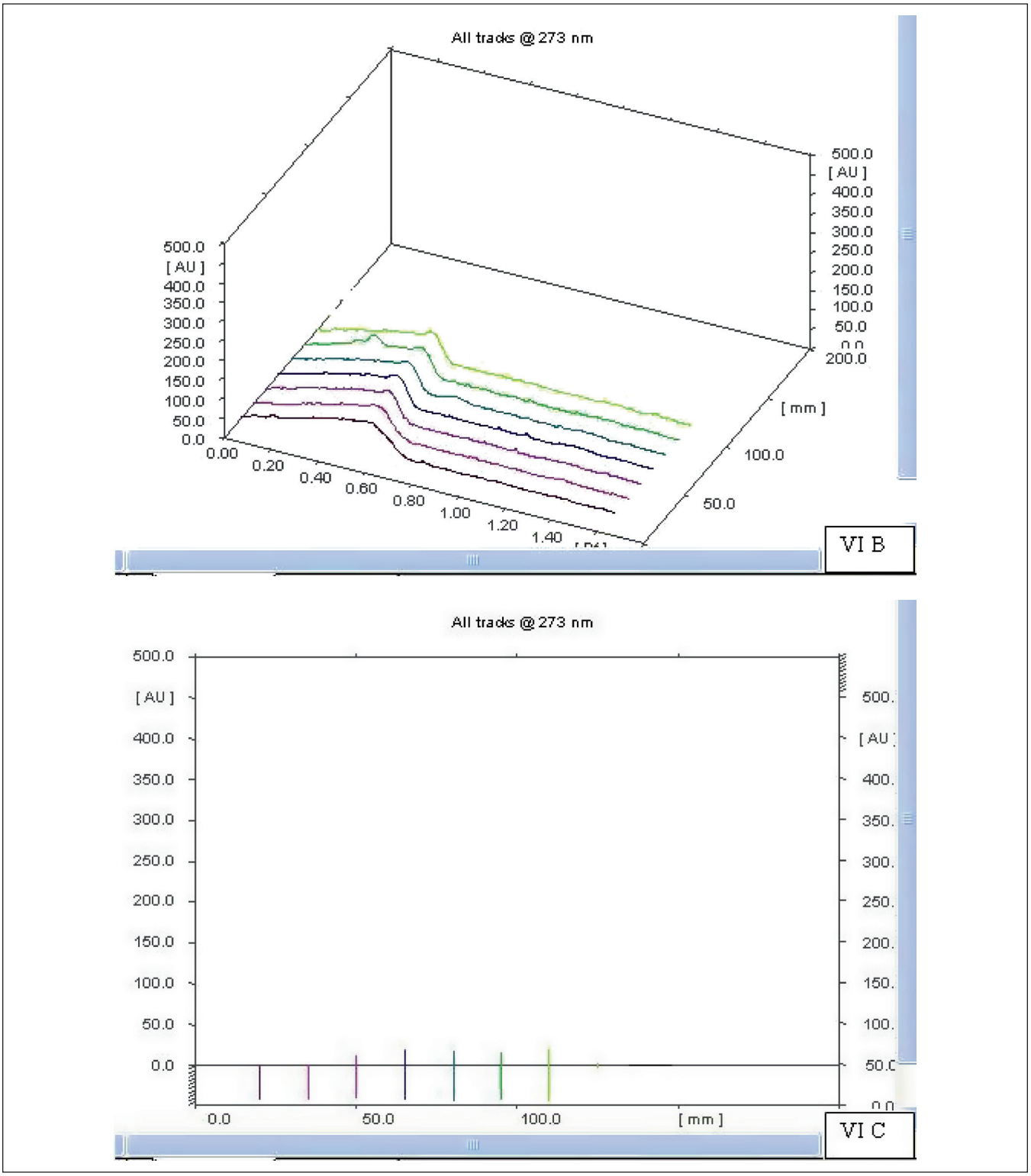

Figure 6: *Continued* (b) 75° (c) 90°

obtained for various tracks are enumerated in Table 3. The calibration curve was linear in the range of 200 to 1000 µg mL^{-1}, as illustrated in Figure 7. From the regression equation, y = 1.483x + 39.67, the concentrations of the test samples i.e. leaves (Track 6) and stem-bark (Track 7) was estimated to be about 791.726 µg mL^{-1} and 696.446 µg mL^{-1} respectively. The estimated value on per gram basis of drug was about 79.172 and 69.644 mg/g of leaves and stem bark respectively.

Table 3: Area under curve values for different concentrations of working standards of β-sitosterol for linear calibration

S.No.	Concentrations of working standard of β-sitosterol (µg mL^{-1})	Area under Curve (AU)
Track 1	200	316.6
Track 2	400	679.3
Track 3	600	977.4
Track 4	800	1268.2
Track 5	1000	1445.7

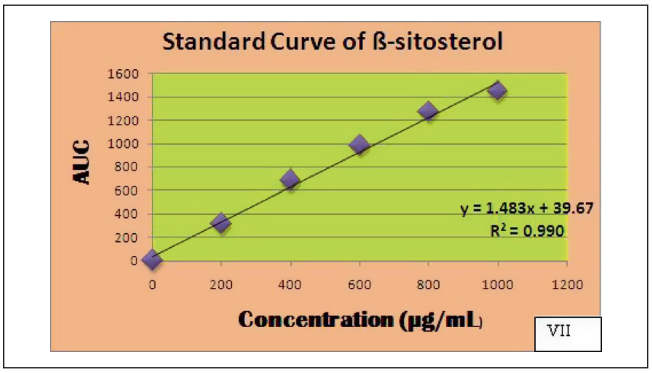

Figure 7: Standard curve (line of best fit) for β-sitosterol

CONCLUSION

The present method provided a quick an easy approach for detection and quantitation of biomarker β-sitosterol in *Michelia champaca* and the estimated values indicate that the leaves are the richest source of the said marker in *M. champaca*. The authors further aim to validate the method in terms of robustness, accuracy and percentage recovery.

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Antihyperglycemic Effect of *Meryta denhamii* Seem. Fruits and Phytochemical study of its Saponin Content

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ABSTRACT

In this study *Meryta denhamii* Seem. fruits (Araliaceae) were tested for the antihyperglycemic effect against alloxan induced hyperglycemia in rats using metformin as standard drug. The alcoholic extract and *n*-butanol fraction (saponins rich fraction) of the fruits exhibited significant antihyperglycemic effect (42.8 and 38.4 % of change, respectively, comparing to 67.1% for metformin). The *n*-butanol fraction was subjected to chemical study which resulted in isolation of four monodesmosidic oleanane saponins. Their structures were established based on their MS, ^{1}H-NMR and ^{13}C-NMR spectral data as 3-*O*-[*β*-D-glucopyranosyl] oleanolic acid, 3-*O*-[*α*-D-glucuronopyranosyl] oleanolic acid, 3-*O*-[*β*-D-glucopyranosyl-(1-3)-*α*-L-arabinofuranosyl] oleanolic acid and 3-*O*-[*α*-L-arabinofuranosyl-(1-4)-*β*-D-glucuronopyranosyl] oleanolic acid.

Key words: *Meryta denhamii* Seem. fruits, triterpenoid saponins, oleanane saponins, antihyperglycemic.

INTRODUCTION

Plants of family Araliaceae are rich in saponin content[1-5]. This class of constituents is characterized by a pronounced molluscicidal activity[6-10]. Antifungal[10-12], antidiabeic[13-15] and antiproliferative[16] activities were also recorded for saponins. *Meryta denhamii* Seem. is an evergreen tree cultivated in public gardens in Egypt, the plant is dioecious, giving globe-like fruits with 12-16 fused berries[17]. The alcoholic extracts of both the flowers and fruits exhibited molluscicidal activity against *Biomphalaria alexandrina* and *Lymnaea Caillaudi*[18], while the alcoholic extract of the stems exhibited anthelmintic activity against adult liver flukes, *Fasciola gigantica*[19]. These observed activities were attributed mainly to the saponin content of the plant. Oleanane saponins were isolated from different organs of the plant[16,18] except the fruits. Thus, this work was conducted on the fruits aiming for testing their antihyperglycemic activity and isolation of these bioactive compounds.

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MATERIAL AND METHODS

General experimental

Mass spectra were performed on UPLC/MS/MS-Waters. NMR spectra were run using Jeol TMS Route instrument at 300 and 90 MHz for measuring ^{1}H and ^{13}C NMR, respectively. TLC was performed on precoated silica gel plates using chloroform: methanol [9:1 (S_1) & 95:5 (S_2)] and chloroform: methanol: formic acid [75:20:5 (S_3)] as solvent systems. The chromatograms were visualized under UV light (at λ_{max} 254 and 366 nm) before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde/sulphuric acid spray reagent.

Plant material

The fruits of *M. denhamii* Seem. were collected from Faculty of Agriculture, Ein Shams University in July, 2011. The plant was kindly authenticated by Mrs T. Labib, taxonomist in El-Orman public garden, Giza, Egypt.

Extraction

About 2 kg of fresh fruits of *M. denhamii* seem. was extracted with cold methanol till exhaustion. After stripping of the solvent under reduced pressure, the residue (100 g) was suspended in water, and then fractionated by successive extraction with suitable volumes of petroleum ether (6 g), chloroform (0.5 g), ethyl acetate (1.2 g) and *n*-butanol (20 g).

Table 1: Antihyperglycemic activity of *Meryta dehamii* Seem. fruits

Groups	Blood glucose level (mg/dl)				
	Zero time	After 4 weeks		After 8 weeks	
	M ± S.E.	M ± S.E.	% of change	M ± S.E.	% of change
Control (1 ml saline)	82.4 ± 1.9	83.6 ± 1.5	1.5	84.3 ± 1.2	2.3
Diabetic non treated	259.4 ± 9.20	262.2 ± 11.3	1.1	265.1 ± 10.4	2.2
Diabetic treated with alcohol extract	249.6 ± 10.3	188.1 ± 9.3*	24.6	142.7 ± 4.9*	42.8
Diabetic treated with *n*-butanol fraction	256.1 ± 11.2	209.4 ± 8.1*	18.2	157.8 ± 5.9*	38.4
Diabetic treated with metformin	264.2 ± 9.3	171.9 ± 6.4*	34.9	86.9 ± 2.3*	67.1

*Statistically significant from control at P < 0.01.

Experimental animals

Sprague Dawley rats (100-150) were obtained from the animal house of National Research Center, Dokki, Giza, Egypt. They were maintained in standard environmental conditions of temperature (25 ± 2 ºC), relative humidity (55 ± 10%) and they were kept in cages and maintained in well ventilated room under natural light and dark cycle.

Drugs and Kits

Alloxan: Sigma Co., Germany.

Glucose Kits: Biomerieux, Germany. Metformin (Cidophage®): Chemical Industries Development Co. (CID Co.), Giza, Egypt.

Antihyperglycemic activity

Rats were divided into five groups (6 animals each), the first group was kept as a control (received 1 ml saline), while for the other groups, diabetes mellitus was induced by intra-peritoneal injection of a single dose of alloxan (150 mg/kg b. wt.) followed by an overnight fasting[20]. A group of diabetic rats was kept non-treated served as negative control, another group received metformin (oral dose of 100 mg/kg b. wt.) as reference drug. The other two groups received the alcoholic extract and *n*-butanol fraction of *M. denhamii* Seem. fruits (oral dose of 100 mg/kg b. wt.). Blood samples were taken at zero time (G_o) and after 4 and 8 weeks (G_t) from the retro-orbital venous plexus, the serum of the blood samples were isolated by centrifugation, then the blood glucose level was estimated using glucose kits according to the method described by Trainder[21]. The percentage of change of blood glucose level was calculated [% of change = $(G_o - G_t) \times 100/G_o$, the data were statistically analyzed using student's t-test[22], the obtained results were given in table 1.

Fractionation and isolation

Ten g of *n*-butanol fraction was fractionated by VLC on silica gel G 60 column (10 × 7 cm). Gradient elution was carried out using chloroform: ethyl acetate mixtures, ethyl acetate and ethyl acetate: methanol mixtures as eluent. Fractions (200 ml each) were collected and monitored by TLC, similar fractions were collected together. Fractions eluted with 100% ethyl acetate, 1% methanol and 5% methanol were pooled and rechromatographed on silica gel column using solvent system chloroform: methanol (95:5) and then, purified on sephadex LH-20 using methanol as eluent to yield compounds **1** and **2**. Fraction eluted with 10% methanol was rechromatographed on silica gel column using solvent system chloroform: methanol (9:1) and then, sephadex LH-20 using methanol: water (1:1) as eluent which afforded compounds **3** and **4**.

Table 2: ^{13}C NMR spectral data of compounds 1, 2, 3 and 4 (δ ppm)

Carbon no.	1	2	3	4
Aglycone				
3	80.04	87.71	87.29	87.69
12	120.40	123.41	120.93	120.70
13	144.97	144.21	144.54	144.50
23	30.37	28.75	28.93	28.92
24	17.21	14.37	16.91	16.39
25	15.49	11.41	15.44	15.08
26	17.36	17.78	17.49	20.57
27	25.41	27.25	26.48	25.43
28	-	-	181.31	-
29	32.08	31.39	32.82	27.60
30	24.98	24.98	23.35	23.45
Sugar(s)	**Glc**	**Glucur**	**Ara**	**Glucur**
1`	104.45	105.47	108.62	105.68
2`	67.34	75.28	74.05	75.59
3`	71.09	79.73	81.02	77.74
4`	64.70	69.14	70.29	83.04
5`	72.64	76.93	67.93	77.54
6`	62.82	174.93		177.30
			Glc	**Ara**
1``			102.34	109.26
2``			74.41	78.77
3``			77.27	76.85
4``			72.22	87.69
5``			77.27	63.66
6``			60.21	

-: not detected

Compound 1: White powder, R_f 0.693 (S_1), MS *m/z*: 617 $[M]^-$, ^{1}H-NMR (300 MHz, DMSO): δ_H 0.58, 0.76, 0.84, 0.86, 0.88, 1.07 and 1.23 (each 3H, s, 7 CH_3), 5.08 (1H, broad s, H-12), 4.23 (1H, broad s, H-1`) and 3.07 - 3.62 (sugar protons). ^{13}C-NMR (90 MHz, DMSO), see table 2.

Compound 2: White powder, R_f 0.671 (S_1), MS *m/z*: 631 $[M]^-$, ^{1}H-NMR (300 MHz, CD_3OD): δ_H 0.77, 0.80, 0.88, 0.90, 0.91, 0.95 and 0.97 (each 3H, s, $7CH_3$), 5.29 (1H, broad s, H-12), 4.46 (1H, broad s, H-1`), 3.54 - 4.46 (sugar protons). ^{13}C-NMR (90 MHz, CD_3OD), see table 2.

Compound 3: White powder, R_f 0.437 (S_1), MS *m/z*: 749 $[M]^-$, ^{1}H-NMR (300 MHz, DMSO): δ_H 0.53 (3H, s, CH_3), 0.72 (3H, s, CH_3), 0.87 (6H, s, $2CH_3$), 1.09 (3H, s, CH_3), 1.23 (6H, s, $2CH_3$), 5.38 (1H, broad s, H-12), 4.54 (1H, broad s, H-1`), 5.14 (1H, broad s, H-1``) and 3.05 - 4.38 (sugars protons). ^{13}C-NMR (90 MHz, DMSO), see table 2.

Compound 4: Needle crystals, R_f 0.166 (S_1), MS *m/z*: 763 $[M]^-$, ^{1}H-NMR (300 MHz, DMSO): δ_H 0.74, 0.85, 0.86, 0.95,1.07 (each 3H, s, $5CH_3$), 1.22 (6H, s, $2CH_3$), 5.10 (1H, broad s, H-12), 4.47 (1H, broad s, H-1`), 4.78 (1H, broad s, H-1``) and 3.03 - 4.11 (sugars protons). ^{13}C-NMR (90 MHz, DMSO), see table 2.

RESULTS AND DISCUSSION

Both the alcoholic extract and *n*-butanol fraction of *M. denhamii* Seem. exhibited significant antihyperglycemic activity (42.8 and 38.4 % of change after 8 weeks, respectively) against alloxan induced hyperglycemia in rats compared to metformin (67.1 % of change after 8 weeks).

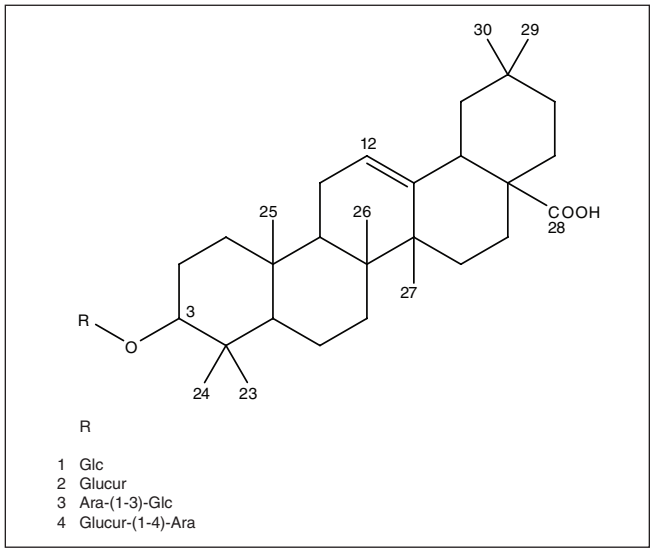
Four triterpenoidal saponins were isolated from the *n*-butanol fraction of *Meryta denhamii,* Seem. fruits by chromatographic fractionation on silica gel and sephadex columns.

^{1}H-NMR of compound **1** displayed seven singlets at δ 0.58, 0.76, 0.84, 0.86, 0.88, 1.07 and 1.23 corresponding to seven tertiary methyls and a trisubstituted olefenic proton (δ 5.08) which are characteristic for oleanane-type triterpene[3]. Signals at δ_H 4.23 and δ_C 104.45 revealed the presence of a sugar molecule. By comparing the spectral data of compound **1** with the published data[18], it was identified as 3-*O*-[α-D-glucopyranosyl] oleanolic acid. This compound was previously isolated from the flowers of the same plant[18].

^{1}H and ^{13}C-NMR of compounds **2, 3** and **4** showed signals for the aglycone part resembling those of compound **1**. Compound **2** have anomeric signals of a sugar molecule at δ_H 4.46 and δ_C 105.47. Signal at δ_C 174.93, corresponding to COOH[4,7,23] group of glucuronic acid, support the presence of glucuronic acid as the sugar molecule. Thus compound **2** was identified as 3-*O*-[α-D- glucuronopyranosyl] oleanolic acid[4,7,23].

^{1}H and ^{13}C NMR of compounds **3** and **4** revealed the presence of two sugar molecules in each compound. The anomeric signals of compound **3** appeared at δ_H 4.54, δ_H 5.14, δ_C 102.34 and δ_C 108.62. By comparing the spectral data of compound **3** with the published data[1,16,24], it was identified as oleanolic acid 3-*O*-[β-D-glucopyranosyl-(1-3)-α-L-arabinofuranosyl].

Compound **4** displayed signals of two anomeric protons at δ_H 4.47 and 4.78 and two anomeric carbons at δ_C 105.68 and 109.26. Compound **4** was identified as 3-*O*-[α-L-arabinofuranosyl-(1-4)-β-D-glucuronopyranosyl] oleanolic acid by comparing its spectral data with the published data[6]. Compounds **2, 3** and **4** were for the first time isolated from this plant. The identity of the four compounds was further confirmed by acid hydrolysis[6] and comparison with reference materials.



CONCLUSION

Triterpenoidal saponins were reported to possess hypoglycemic activity[13-15], thus the observed antihyperglycemic activity of the *n*-butanol fraction could be attributed mainly to its saponin content. Other plant constituent viz. flavonoids also possess antihyperglycemic activity[25-27], this could explain the higher activity of the alcoholic extract compared to the *n*-butanol fraction.

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Antimicrobial Activity of the Crude Extracts of *Withania somnifera* and *Cenchrus setigerus In-vitro*

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ABSTRACT

Introduction: The aim of present study is to investigate the antimicrobial activity of *Withania somnifera* (RUBL-20668) and *Cenchrus setigerus* (CAZRI-76) extracts in order to use it as a possible source for new antimicrobial substances against important human pathogens. **Method:** The crude extracts of different parts (root, stem, leaf and seed) of *C. setigerus* and (Unripen fruit, ripen fruit, calyx) of *W. somnifera* and were successively extracted with polar to non polar solvents using soxhlet assembly. The extracts were then screened for their antimicrobial activity *in-vitro* against one gram positive bacteria (*Bacillus subtilis*), two gram negative bacteria (*Pseudomonas aeruginosa* and *Enterobactor aerogens*) and one fungus (*Aspergillus flavus*) by disc diffusion assay. Serial dilution method was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC). **Result:** Water extract of stems of *C. setigerus* (IZ-21.83 ± 0.24 mm, AI- 0.780) and chloroform extract of calyx of *W. somnifera* (IZ-16.17 ± 0.24 mm, AI- 1.078) showed highest activity against *B. subtilis*. **Conclusion:** The demonstration of broad spectrum of *W. somnifera* and *C. setigerus* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control.

Key words: *Aspergillus flavus, Bacillus subtilis, Cenchrus setigerus, Enterobactor aerogens, Pseudomonas aeruginosa, Withania somnifera*.

INTRODUCTION

Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanism of action.[1-4] Plant extract has been used traditionally to treat a number of infectious diseases including those caused by bacteria and fungi.[5-7] A number of reports are available *in-vitro* and *in-vivo* efficacy of plant extract against plant and human pathogens causing fungal infections,[8] but more study and screen is required because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Therefore, there is a need to look for substances from other sources with proven antimicrobial activity.

C_4 grasses are more competitive under the conditions of high temperature, solar radiation and low moisture.[9] C_4 grasses are more efficient at gathering Carbon dioxide, utilizing nitrogen from the atmosphere and recycled N in the soil.[10-11] *C. setigerus* L. (Poaceae) is highly nutritious grass and considered excellent for pasture in hot, dry areas and is valued for its production of palatable forage and intermittent grazing during droughty periods in the tropics. This grass has excellent soil binding capacity which helps to conserve soil in desert areas.[12] Although, *C. setigerus* is most suitable and highly nutritive grasses for desert environmental conditions, still no antimicrobial work has yet been recorded.

The present investigation evaluated the antibacterial and antifungal effects of crude extracts of *C. setigerus* and

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W. somnifera. The study was carried out along with the standard drugs Gentamycin (for bacteria), Ketoconazole (for fungi).

MATERIAL AND METHODS

Experimental design: Crude extracts of different parts of *W. somnifera* (RUBL-20668) and *C. setigerus* (CAZRI-76) were prepared with a series of non polar to polar solvents by hot extraction method[13] in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay[14] against a few medically important bacteria and fungi. The fraction showing best activity was then used for determining minimum inhibitory concentration (MIC) by serial dilution method[15] and minimum bactericidal/fungicidal concentration (MBC/MFC).

Collection of plant material: Different parts of *C. setigerus* (CAZRI-76) were collected in the month of August from the Central Arid Zone Research Institute, Jodhpur, Rajasthan and parts of *W. somnifera* (RUBL-20668) were collected in the month of January from Jaipur district of Rajasthan. *W. somnifera* was identified by the herbarium, department of botany, university of Rajasthan, Jaipur. The collected plant materials were transferred immediately to the laboratory cleaned with water and selected plant parts were separately shade dried for one week. Each shade dried plant part was powdered with the help of grinder. Fine powder of each sample was stored in clean container to be used for Soxhlet extraction following the method of Subramanian and Nagarjan[16] in different polar solvents selected.

Extraction procedure: Each plant part (10 gm) was sequentially extracted with different solvents (250 ml) according to their increasing polarity (Benzene < Chloroform < Water) by using Soxhlet apparatus for 18 hours at a temperature not exceeding the boiling point of the respective solvent. The obtained extracts were filtered by using Whatman No. 1 filter paper. The extracts solutions were evaporated under reduced pressure at 40 °C.[17] The residual extracts were stored in refrigerator at 4 °C in small and sterile glass bottles. Percent extractive values were calculated by the following formula (table-3).

$$\text{Percent Extracts} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100$$

Drugs and chemicals used

Drugs: Gentamycin (for bacteria) and Ketoconazole (for fungi)

Chemicals: Benzene, Chloroform, Water, Nutrient Agar (for bacteria) Sabouraud Dextrose Agar (for fungi).

Micro-organisms

(a) Bacteria: *Pseudomonas aeruginosa* (G-ve) (MTCC-1934), *Bacillus subtilis* (G+ve) (MTCC-121), *Enterobactor aerogens* (G-ve) (MTCC-111)

(b) Fungi: *Aspergillus flavus* (MTCC-277).

Screening for antimicrobial activity: Test pathogenic microorganisms were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on Nutrient Agar medium, while fungus was maintained on Sabouraud Dextrose Agar medium. Disc diffusion assay[14] was performed for screening. Sterile filter paper discs (Whatman no. 1, 5 mm in diameter) were impregnated with 100 µl of each of the extract (10 mg/ml) to give a final concentration of 1 mg/disc and left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. A bacterial suspension was prepared and added inoculum size 1×10^8 CFU/ml for bacteria and 1×10^7 cell/ml for fungi[18] to the sterilized medium before solidification. The media with bacteria was poured into sterilized Petri dishes under aseptic condition.[19] Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate with gentamycin (10mcg/disc) and ketoconazole (10 mcg/disc) as standard for bacteria and fungi, respectively. The plates were kept at 4 °C for 1 h for diffusion of extract, thereafter were incubated at 37 °C for bacteria (24 h) and 27 °C for fungi (48 h).[20] After incubation the average of inhibition zones was recorded.[21-22] Inhibition zones were measured and compared with the standard reference antibiotics Activity index for each extract was calculated (Table 1).

$$\text{Activity index (AI)} = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}$$

Determination of minimum inhibitory concentration (MIC): The Minimum inhibitory concentration (MIC) value was considered as the lowest extract concentration with no visible growth for each plant extract test pathogens.[20] To measure the MIC values, various concentrations of the stock, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.234, 0.117, 0.059, 0.029 mg/ml were assayed against the test pathogens. Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 15 mg/ml final concentration and then two fold serially diluted; 1 ml of each extract was added to test tubes containing 1 ml of sterile Nutrient Agar media (for bacteria) Sabouraud Dextrose Agar media (for fungi). The tubes were then inoculated with standard size of microbial suspension (for bacteria 1×10^8 CFU/ml and 1×10^7 cell/ml for fungi) and the tubes were incubated at 37 °C for 24 h for bacteria and 28 °C for 48 h for fungi in a BOD incubator and observed for change in turbidity after 24 h and compared with the growth in controls.[23] A tube containing nutrient

broth and inoculum but no extract was taken as control. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Each extract was assayed in duplicate and each time two sets of tubes were prepared, one was kept for incubation while another set was kept at 4 °C for comparing the turbidity in the test tubes.

Determination of Minimum bactericidal/fungicidal concentration (MBC/MFC): Equal volume of the various concentration of each extract and nutrient agar were mixed in micro-tubes to make up 0.5 ml of solution. Then 0.5 ml of McFarland standard of the organism suspension was added to each tube.[24] The tubes were incubated aerobically at 37 °C for 24 h for bacteria and 28 °C for 48 h for fungi. Two control tubes were maintained for each test batch. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Nutrient Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration.[25] MBC was calculated for some of the extracts showed high antimicrobial activity against highly sensitive organisms.

Total activity (TA) determination: Total activity is the volume at which the test extract can be diluted with the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.[26]

$$\text{Total Activity} = \frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$$

Statistical Analysis: Mean value and standard deviation were calculated for each test bacteria and fungi. Data were analyzed by one-way ANOVA and p values were considered significant at $p > 0.005$.[27]

RESULTS

Preliminary phyto–profiling: The preliminary phyto–profiling for the different parts of *W. somnifera* (RUBL-20668) and *C. setigerus* (CAZRI-76) were carried out according to Farnsworth,[28] where in the consistency was found to be sticky in the chloroform solvent extracts of both plants. The highest yield was recorded for Unripen fruit extracts of *W. somnifera* in water (21.73%) and 4.19% for leaf extracts of *C. setigerus* in chloroform solvent. (Table 3)

Table 1: Inhibition zone (mm)* and Activity index of different parts of *Cenchrus setigerus* and *Withania somnifera* against pathogens

Solvents	Polarity of Solvents	Plant Part	Test microorganisms							
			Cenchrus setigerus (CAZRI-76)							
			Pseudomonas aeruginosa		*Bacillus subtilis*		*Enterobactor aerogens*		*Aspergillus flavus*	
			IZ ± S.D.	AI	IZ ± S.D.	AI	IZ ± S.D.	AI	IZ ± S.D.	AI
Benzene	2.7	Root	-	-	-	-	-	-	-	-
		Stem	8.17 ± 0.23	0.454	-	-	-	-	-	-
		Leaf	-	-	-	-	-	-	-	-
		Seed	-	-	-	-	-	-	-	-
Chloroform	4.1	Root	-	-	-	-	-	-	-	-
		Stem	-	-	12.5 ± 0.64	0.521	8.5 ± 0.64	0.567	-	-
		Leaf	-	-	9.33 ± 0.24	0.389	-	-	-	-
		Seed	-	-	8.17 ± 0.23	0.340	10.67 ± 0.24	0.711	-	-
Water	9	Root	-	-	9.17 ± 0.23	0.328	13.67 ± 0.26	0.684	-	-
		Stem	-	-	21.83 ± 0.24	0.780	10.33 ± 0.23	0.517	-	-
		Leaf	-	-	8.67 ± 0.23	0.310	12.67 ± 0.24	0.634	-	-
		Seed	-	-	9.17 ± 0.26	0.328	11.5 ± 0.64	0.575	-	-
			Withania somnifera (RUBL-20668)							
Benzene	2.7	UnF	-	-	-	-	-	-	-	-
		RpF	-	-	-	-	-	-	-	-
		Calyx	-	-	-	-	-	-	-	-
Chloroform	4.1	UnF	8.50 ± 0.64	1.063	10.17 ± 0.21	0.678	-	-	-	-
		RpF	7.17 ± 0.29	0.896	8.33 ± 0.23	0.555	-	-	-	-
		Calyx	-	-	16.17 ± 0.24	1.078	-	-	-	-
Water	9	UnF	9.17 ± 0.28	1.146	-	-	-	-	-	-
		RpF	8.33 ± 0.24	1.041	-	-	-	-	-	-
		Calyx	10.5 ± 0.64	1.313	-	-	-	-	-	-

*All values are mean ± SD; n = 3 ($p > 0.005$); UnF- Unripen Fruit; RpF- Ripen Fruit

Antimicrobial activity: Antimicrobial activity (denoted in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded (Table 1). In the present study total 21 extracts of different parts of selected plants were tested for their bioactivity. Fourteen extracts showed significant antimicrobial potential against test microbes. However 7 extracts showed no activity against any of the selected microorganisms at the tested concentration (four from *C. setigerus* and three from *W. somnifera*). Most susceptible organism in the investigation was *B. subtilis* against which, most of the plant extracts showed inhibition zone. Maximum Antimicrobial activities were recorded for stem of *C. setigerus* in water extracts against *B. subtilis*.

Antibacterial activity: Stem extracts of *C. setigerus* showed maximum antibacterial activity in water solvent (IZ- 21.83 ± 0.24 mm, AI-0.780) followed by calyx extracts of *W. somnifera* in chloroform solvent (IZ- 16.17 ± 0.24 mm, AI-1.078) against *B. subtilis*. Benzene extracts of *W. somnifera* didn't show any antibacterial activity.

Antifungal activity: All the extracts of both the plants did not show any antifungal activity against *A. flavus*. It indicate that *A. flavus* is highly resistant pathogen.

MIC and MBC/MFC: MIC and MBC/MFC values (Table 2) were recorded for those plant extracts only, which shows activity in disc diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.938-15 mg/ml. In the present investigation lowest MIC values were recorded for *C. setigerus* leaf and stem extracts in water solvent (0.938 mg/ml) against *E. aerogens* and *B. subtilis* respectively as well as for *W. somnifera* unripen fruit and calyx extracts in chloroform solvents against *B. subtilis* indicating significant antimicrobial potential of test extracts.

Total activity: Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganism (Table 3). Most of the extracts of *W. somnifera* showed high values of TA against *P. aeruginosa* and *B. subtilis*, which proves the potential of extracts to inhibit growth of the test microorganisms, even at low concentration. In *W. somnifera* maximum TA values were calculated in water solvent, for unripen fruit extracts (57.96 ml) followed by calyx extracts (55.58 ml) and ripen fruit (54.33 ml) against *P. aeruginosa*. In *C. setigerus* maximum TA values were calculated for leaf (21.01 ml) and stem extracts (12.69 ml) in water solvent against *E. aerogens* and *B. subtilis* respectively.

Table 2: Showing minimum inhibitory concentration and (MBC/MFC) of different parts of *Cenchrus setigerus* and *Withania somnifera* against pathogens

Solvents	Plant Part	Test microorganisms							
		***Cenchrus setigerus* (CAZRI-76)**							
		P. aeruginosa		***B. subtilis***		***E. aerogens***		***A. flavus***	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
Benzene	Root	-	-	-	-	-	-	-	-
	Stem	1.875	3.75	-	-	-	-	-	-
	Leaf	-	-	-	-	-	-	-	-
	Seed	-	-	-	-	-	-	-	-
Chloroform	Root	-	-	-	-	-	-	-	-
	Stem	-	-	1.875	3.75	7.5	15	-	-
	Leaf	-	-	3.75	7.5	-	-	-	-
	Seed	-	-	-	-	3.75	3.75	-	-
Water	Root	-	-	7.5	15	1.875	1.875	-	-
	Stem	-	-	0.938	1.875	1.875	3.75	-	-
	Leaf	-	-	7.5	15	0.938	1.875	-	-
	Seed	-	-	7.5	15	1.875	3.75	-	-
		***Withania somnifera* (RUBL-20668)**							
Benzene	UnF	-	-	-	-	-	-	-	-
	RpF	-	-	-	-	-	-	-	-
	Calyx	-	-	-	-	-	-	-	-
Chloroform	UnF	3.75	3.75	0.938	1.875	-	-	-	-
	RpF	7.5	15	3.75	7.5	-	-	-	-
	Calyx	-	-	0.938	1.875	-	-	-	-
Water	UnF	3.75	7.5	-	-	-	-	-	-
	RpF	3.75	3.75	-	-	-	-	-	-
	Calyx	3.75	3.75	-	-	-	-	-	-

UnF- Unripen Fruit; RpF- Ripen Fruit; MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration; MFC- minimum fungicidal concentration

Table 3: Preliminary phyto-profile for different parts of *C. setigerus* and *W. somnifera* against pathogens in different polar solvents

Solvents	Parts	% Yield	Color	Consistency	Total activity of microorganisms			
					P. a.	*B. s.*	*E. a.*	*A. f.*
***Cenchrus setigerus* (CAZRI-76)**								
Benzene	R	0.87	Yellow	Nonsticky	-	-	-	-
	S	1.93	Yellow	Nonsticky	10.29	-	-	-
	L	1.79	Dark brown	Sticky	-	-	-	-
	Se	1.21	Yellow	Sticky	-	-	-	-
Chloroform	R	3.22	Brown	Sticky	-	-	-	-
	S	3.57	Greenish brown	Sticky	-	19.04	4.76	-
	L	4.19	Green	Sticky	-	11.17	-	-
	Se	3.89	Yellow	Sticky	-	-	10.37	-
Water	R	2.15	Dark brown	Nonsticky	-	2.87	11.47	-
	S	1.19	Brick red	Nonsticky	-	12.69	6.35	-
	L	1.97	Dark coffee	Sticky	-	2.63	21.01	-
	Se	2.02	Dark brown	Nonsticky	-	2.69	10.77	-
***Withania somnifera* (RUBL 20668)**								
Benzene	UnF	6.39	Black	Oily	-	-	-	-
	RpF	7.03	Yellow brown	Oily	-	-	-	-
	Calyx	4.24	Light green	Oily	-	-	-	-
Chloroform	UnF	2.62	Dark green	Sticky	7.00	27.99	-	-
	RpF	12.77	Yellow	Sticky	17.03	34.06	-	-
	Calyx	3.03	Parrot green	Sticky	-	32.31	-	-
Water	UnF	21.73	Light yellow	Nonsticky	57.96	-	-	-
	RpF	20.38	Light yellow	Nonsticky	54.33	-	-	-
	Calyx	20.84	Light green	Nonsticky	55.58	-	-	-

R- Root; S-Stem; L- Leaf; Se- Seed; UnF- Unripen Fruit; RpF- Ripen Fruit; *P. a. - Pseudomonas aeruginosa*; *B. s. – Bacillus subtilis*; *E. a. - Enterobactor aerogens*; *A. f. - Aspergillus flavus*

DISCUSSION

Results of the present study reveled that 14/21 plant extracts tested, inhibited the growth of selected bacteria and fungi, indicating broad spectrum bioactive nature of selected two plants (8/12 in *C. setigerus* and 6/9 in *W. somnifera*). It indicates that *C. setigerus* is more potential than *W. somnifera* as far as bioactivity concerned. In general, stem extracts of *C. setigerus* and calyx extracts of *W. somnifera* express maximum antibacterial activities by suppressing the growth of microbes under investigation. Excellent antibacterial activities were observed for water extracts of *C. setigerus* and chloroform extracts of *W. somnifera* indicated due to low MIC and MBC/MFC values. MBC/MFC values were found higher than the MIC values of the extracts against microorganisms tested; indicate the bacteriostatic/fungistatic effects of the extracts. Water extracts of root in *C. setigerus* were recorded as bactericidal against *E. aerogens*. Gram positive bacteria *B. subtilis* was the most susceptible organism which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative.[29-30] Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria.

Extracts under study not only inhibit the bacterial growth but the IZ developed, was more or less permanent when compared with the IZ developed by the standard drug used, as after sometime bacterial colonies could be easily seen in IZ developed by standard drugs. In the light of the fact that microorganism are becoming resistant against the drugs in use, present investigation is of great significance, as far as the future drugs are concerned and choice of selected plants by the pharmaceutical industries for preparing plant based antimicrobials drugs.

C. setigerus easily grows in harsh climatic conditions or xeric conditions and requires less care; hence its use as raw material for preparing drugs would definitely be economical.

CONCLUSION

In the present study total 21 extracts of different parts of desert grasses were tested for their bioactivity, among which 14 extracts showed significant antibacterial potential against test microbes. Compared to reference antibiotics, the spectrum of antibacterial activity of investigated plants was found superior. The demonstration of broad spectrum of *W. somnifera* and *C. setigerus* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. The effect of these plants on more pathogenic organisms, and toxicological investigations and further purification, however, need to be carried out.

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Antihyperlipidemic Activity of Flowers of *Punica granatum* in Poloxamer-407 Induced Hyperlipidemic Mice Model

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ABSTRACT

Introduction: The methanolic partitionate of pet ether extract of flowers of *Punica granatum* (Family: Punicaceae) was evaluated for antihyperlipidemic activity in poloxamer 407 induced hyperlipidemic mice. *Methods:* Hyperlipidemia was induced in mice by i.p. injection of poloxamer 407 (30% w/w in distilled cool water; 600 mg/kg) and 2 hours after the administration of P-407, the mice of reference group were administered with atorvastatin (50 mg/kg p.o.), while test group received the methanolic partitionate of pet ether extract of flowers of *Punica granatum* (500 mg/kg p.o). After 15 and 24 hour of treatment, serum lipid profiles were investigated using commercially available kits. *Results:* The administration of the flower extracts significantly ($p < 0.05$) reduced the serum levels of triglycerides (TG) and very low density lipoprotein (VLDL) as well as the atherogenic index (A.I.) and significantly increased the serum high density lipoprotein (HDL) level compared to the P-407 induced hyperlipidemic control mice after 15 h of treatment at a single dose of 500 mg/kg p.o. After 24 h of treatment, the extract induced a significant reduction ($p < 0.05$) in serum total cholesterol (TC), VLDL, low density lipoprotein (LDL) as well as the atherogenic index and significant increase in HDL levels, when compared to P-407 control group. All these effects were comparable to those of the reference standard, atorvastatin. *Conclusions:* The results of the investigation demonstrated that the flower extract of *Punica granatum* has potential antihyperlipidemic activity and might be used for the prevention of hyperlipidemia associated disorders.

Key words: atherogenic index**;** hyperlipidemia**;** lipid profile**;** methanolic partitionate; pet ether extract; serum

INTRODUCTION

Punica granatum Linn. (Punicaceae), commonly called pomegranate, is an attractive large shrub or small tree native from Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe.[1] The most important growing regions are Egypt, China, Afghanistan, Pakistan, Bangladesh, Iran, Iraq, India, Burma and Saudi Arabia.

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Pomegranate is an important medicinal plant containing versatile bioactive compounds and traditionally was used in the treatment of different diseases. The ripe fruit is tonic, astringent, laxative and diuretic, used in brain diseases, chest troubles, bronchitis and earache. Bark and fruit rind are administered orally to prevent dysentery, diarrhea, piles, bronchitis, biliousness and as an anthelmintic.[2] The dried flowers, known as Gulnar, are efficaceous to treat hematuria, hemoptysis, diarrhea, dysentery, bronchitis, nasal hemorrhage.[3] Pomegranate flowers have been prescribed in Unani and Ayurvedic medicines for the treatment of diabetes.[4] It has been demonstrated that flower extract shows hypoglycemic activity in normal and alloxan-induced diabetic animals.[5] Flower juice is recommended as a gargle for sore throat, oral and throat inflammation, in leucorrhea, hemorrhage and ulcers of the uterus and rectus.[3]

Some studies have reported that both the pomegranate flowers and fruit extracts exhibited high activity on lowering

circulation lipid and modifying heart disease risk factors in diabetic animals and humans with hyperlipidemia.[6-8] Lei et al.[8] reported that the pomegranate leaf extract containing abundant tannins, had a strong lipid-lowering action in hyperlipidemic animals after a long-term of oral administration. Huang et al.[9] investigated the effects of flower extract of *Punica granatum* extract on abnormal cardiac lipid metabolism both in vivo and in vitro and reported that it reduced cardiac TG content, accompanied by a decrease in plasma levels of TG and total cholesterol in Zucker diabetic fatty (ZDF) rats. Priyanka et al.[2] demonstrated that oral administration of aqueous extract of flower of *Punica granatum* significantly reduced fasting blood glucose and serum lipid profile in streptozotocin induced diabetic rats.

As the dried flowers of *Punica granatum* have been prescribed in Unani and Ayurvedic medicines for the treatment of diabetes and as all the previous investigations reported the lipid lowering activity of flowers of *P. granatum* in diabetic rat model, our present study was designed to investigate the antihyperlipidemic activity of methanolic fraction of petroleum ether extract of flowers of *Punica granatum* in Poloxamer-407 induced hyperlipidemic mice model.

MATERIALS AND METHODS

Chemicals and reagents

Poloxamer 407, also called Pluronic RF-127, was donated by BASF Bangladesh Ltd. Atorvastatin tablet at a strength of 40 mg was collected from Beximco Pharmaceuticals Ltd. Bangladesh. Total cholesterol (TC), total triglyceride, (TG), high density lipoprotein cholesterol (HDL) measuring kits were purchased from Linear Chemicals S.L. (Barcelona, Spain). Dimethylsulfoxide and Tween-80 were purchased from Sigma-Aldrich . All other reagents and chemicals were of BDH and E-Merck analytical grade.

Preparation of plant extract

The flower of the plant *Punica granatum* was collected from Naogaon in February, 2010 and authentification of the sample was confirmed by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen was deposited (accession number: DACB 35050) in the Herbarium for further reference. The collected flowers were washed, cut into small pieces and dried in the sun for about a week. After drying, the plant materials were kept in an oven at 40 °C to ensure complete drying. The dried samples were then ground in coarse powder using high capacity grinding machine. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use. About 500 g of powdered flower material was taken in clean, round bottomed flask (5 liters) and macerated with 2 liters of petroleum ether at room temperature for 7 days with occasional shaking. The whole mixture was then filtered through cotton plug followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 40 °C under reduced pressure with a Heidolph rotary evaporator. The concentrated extract was then air dried to solid residue. The weight of the crude pet-ether extract was 70.5 g. Solvent–solvent partitioning was done using the protocol designed by Kupchan[10] and modified version of Wagenen et al.[11] The crude extract (5 gm) was dissolved in 10% aqueous methanol which was subsequently extracted with petroleum ether, dichloromethane and methanol. All the three partitioning (pet ether fraction, dichloromethane fraction and methanolic fraction) fractions were evaporated to dryness by using rotary evaporator and kept in air tight containers for further analysis. The methanolic partitionate and standard drug atorvastatin were suspended in normal saline using 0.1% tween-80.

Experimental animals

Swiss albino mice of either sex, weighing 35-40 g were purchased from the animal resource branch of International Center for Diarrheal Diseases and Research, Bangladesh (ICDDR,B). The animals were kept in standard environmental conditions (temperature: 23 ± 2 °C; relative humidity: 55 ± 10 % and 12 hours light/ dark cycle). The animals were fed with standard pellets diet (ICDDR,B formulated) and water *ad libitum* and acclimatized to laboratory conditions for 7 days before the experimentation. The design and performance of research study involving mice have been approved by the Ethical Review Committee, Faculty of Biological Science, University of Dhaka through the submission of a research protocol before the study.

Experimental procedures

Poloxamer-407-induced acute hyperlipidemia in mice

The antihyperlipidemic activity was evaluated according to the method described by Hitesh et al.[12] Twenty swiss albino mice were divided into four groups of five mice each (Table I). To render the mice hyperlipidemic, the animals were kept in fasting condition for 6 hours before the experimentation. Based on the reported method[13] the mice of Group II, III and IV were made hyperlipidemic by an intraperitoneal injection of 600 mg/kg of P-407 that had been prepared at a final concentration of 30% (w/w) by dissolving the powder in distilled cool water and the solution was then kept refrigerated overnight to facilitate its dissolution.[14] Two hours after the administration of P-407, the mice of Group III (reference group) were administered with atorvastatin at a dose of 50 mg/kg p.o., while group IV (test group) received the methanolic partitionate of pet ether extract of flowers of *Punica granatum*

at a dose of 500 mg/kg p.o. On the other hand, the normal control group (Group I) received the vehicles (1% Tween-80 in normal saline) at a dose of 10 ml/kg p.o.

Blood sampling

After 15 and 24 h of treatment, blood samples were collected from the retro-orbital plexus of each mouse after they have anesthetized with diethyl ether and the samples were incubated at room temperature for 30 minutes. The blood samples were then centrifuged at 4000 rpm at 4 ºC for 10 min; the serum was separated and stored at –40 ºC until to be used for biochemical tests.

Biochemical estimations of lipid profile

The levels of triglycerides (TG), total cholesterol (TC) and high density lipoprotein cholesterol (HDL-C) in the serum were estimated by enzymatic colorimetric methods using commercial Kits (Linear Chemicals Ltd, Spain) according to manufacturer's instructions.[15-18] Very low–density lipoprotein cholesterol (VLDL-C) was calculated as TG/5. LDL- cholesterol (LDL-C) levels were calculated using Friedewald's formula[19]:

$$\text{LDL-C} = \text{TC-HDL} - (\text{TG}/5).$$

The atherogenic index (A.I) was calculated using the formula:

$$\text{Atherogenic index (A.I)} = \frac{(\text{VLDL-C} + \text{LDL-C})}{\text{HDL-C}}$$

Statistical analysis

The results were expressed as the mean ± SEM (Standard error mean). The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunett's *t* test to determine the level of significance. A value of $p < 0.05$ was considered to be significant. The statistical analysis was carried out using the SPSS program (version 17.0).

RESULTS

The serum TC, TG, LDL-C and VLDL-C levels were significantly ($p < 0.001$) increased in the hyperlipidemic P-407 control group at 15 h (Table I) and 24 h (Table II), when compared with the normal control group. But the methanolic partitionate of pet ether extract of flowers of *P. granatum* (MPPG) was found to be effective in significantly reducing serum TG and VLDL ($p < 0.001$) levels when compared to the P-407 induced hyperlipidemic control

Table I: Effect of methanolic partitionate of flowers of *Punica granatum* (MPPG) on serum lipid profile 15 h after poloxamer 407 -induced acute hyperlipidemia in mice[a]

Group	TC (mmol/L)	TG (mmol/L)	HDL[b] (mmol/L)	LDL (mmol/L)	VLDL (mmol/L)	A.I.
Group-I (Normal control)	3.62 ± 0.42	3.19 ± 0.16	1.13 ± 0.09	2.05 ± 0.39	0.63 ± 0.03	2.42 ± 0.39
Group-II (P-407 control)	18.39 ± 0.63*	16.93 ± 0.75*	5.04 ± 0.20*	9.9 ± 0.67*	3.38 ± 0.08*	2.67 ± 0.27
Group-III (Atorvastatin)	13.47 ± 0.51** (26.75)	9.45 ± 0.40** (44.18)	7.68 ± 0.29** (52.38)	3.9 ± 0.56** (60.60)	1.89 ± 0.08** (44.10)	0.76 ± 0.08** (71.53)
Group-IV (MPPG)	16.9 ± 0.60 (8.10)	10.57 ± 1.17** (37.56)	6.23 ± 0.39*** (23.61)	8.56 ± 0.62 (13.53)	2.11 ± 0.23** (37.57)	1.74 ± 0.16*** (34.83)

All values are expressed as mean ± S.E.M. (n = 5). One way ANOVA followed by Dunnett's ***t*** test. *compared to normal control group (***p*** < 0.001); **compared to P-407 control group (***p*** < 0.001); ***compared to P-407 control (***p*** < 0.05). Figures in parentheses are the percentage reduction compared to P-407 control group. [b]Figures in parentheses are the % increase compared to P-407 control group. [a]TC: total cholesterol, TG: triglyceride, HDL: High-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein, A.I.: atherogenic index.

Table II: Effect of methanolic partitionate of flowers of *Punica granatum* (MPPG) on serum lipid profile 24 h after poloxamer 407 -induced acute hyperlipidemia in mice[a]

Group	TC (mmol/L)	TG (mmol/L)	HDL[b] (mmol/L)	LDL (mmol/L)	VLDL (mmol/L)	A.I.
Group-I (Normal control)	4.21 ± 0.51	3.43 ± 0.13	1.18 ± 0.07	2.46 ± 0.44	0.68 ± 0.02	2.64 ± 0.32
Group-II (P-407 control)	24.28 ± 0.89*	17.66 ± 0.46*	5.05 ± 0.17*	15.7 ± 0.80*	3.52 ± 0.09*	3.82 ± 0.20*
Group-III (Atorvastatin)	14.6 ± 0.44** (39.86)	13.61 ± 0.24** (22.93)	7.00 ± 0.30** (38.61)	4.87 ± 0.69** (68.98)	2.72 ± 0.049** (22.72)	1.1 ± 0.12** (71.20)
Group-IV (MPPG)	19.72 ± 0.67*** (18.78)	14.56 ± 0.46** (17.55)	6.06 ± 0.29*** (20.0)	10.74 ± 0.95*** (31.6)	2.91 ± 0.09** (17.32)	2.3 ± 0.26** (39.79)

All values are expressed as mean ± S.E.M. (n = 5). One way ANOVA followed by Dunnett's *t* test. *compared to normal control group (*p* < 0.001); **compared to P-407 control group (*p* < 0.001); ***compared to P-407 control (*p* < 0.05). Figures in parentheses are the percentage reduction compared to P-407 control group. [b]Figures in parentheses are the % increase compared to P-407 control group. [a]TC: total cholesterol, TG: triglyceride, HDL: High-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein, A.I.: atherogenic index.

mice, after 15 and 24 h of treatment at a dose of 500 mg/kg p.o. (Table I and Table II). After 15 h of treatment, MPPG reduced serum TC and LDL-C levels but the results were not found to be significant but after 24 h of treatment, both the parameters were reduced significantly ($p < 0.05$). MPPG also increased the serum HDL-C levels significantly ($p < 0.05$) after 15 and 24 h of treatment, when compared to the P-407 control group. The most useful finding was that MPPG significantly lowered the atherogenic index (A.I.) after 15 h ($p < 0.05$) and 24 h ($p < 0.001$) of treatment. All these effects were comparable to those of the reference standard, atorvastatin.

DISCUSSION

Hyperlipidemia characterized by abnormally elevated serum triacylglycerol (TG), total cholesterol (TC), LDL-C and VLDL-C, is an established risk factor for the development of coronary artery disease (CAD).[20] In the present study, the effects of methanolic partitionate of pet-ether extract of flowers of *P. granatum* on serum lipid levels was evaluated in hyperlipidemic mice induced by poloxamer 407. P-407 has been utilized in the hyperlipidemic model due to its convenience, reproducibility and the lack of undesirable underlying pathological conditions.[21] Increased serum total cholesterol (TC), triglycerides (TG) and low density lipoprotein cholesterol (LDL-C) levels are important risk factors for atherosclerosis development.[22-24] On the other hand, elevated levels of HDL-C exert an anti-atherogenic effect by counteracting LDL-C oxidation and facilitating the translocation of cholesterol from peripheral tissue such as arterial walls to the liver for catabolism.[25] The A.I., the ratio of LDL to HDL, is commonly used as an index for atherosclerosis.[26] Treatment of mice with methanolic partitionate of pet-ether extract of *Punica granatum* significantly reduced the serum TG, TC, VLDL and LDL levels as well as the A.I and significantly increased the serum HDL-C levels when compared to hyperlipidemic control mice (Table I and II). The results imply that oral administration of methanolic extract of flowers of *P. granatum* have potential ability to reduce the risk of atherosclerosis.

The serum TG lowering activities of methanolic partitionate of pet-ether extract of *Punica granatum* flower can be attributed to the ability of the extract to increase the lipoprotein lipase activities since Johnston and Plamer[13] and Johnston[20] have demonstrated that the increase in triglycerides (TG) mediated by P-407 ip. injection to rats results primarily from an inhibition of TG degradation, where P-407 directly inhibits capillary lipoprotein lipase (LPL) responsible for plasma TG hydrolysis. The cholesterol lowering effects of the extracts of the plant might be due to the inhibition of hepatic HMG CoA reductase, the rate-limiting enzyme in the biosynthesis of cholesterol since atorvastatin which was used as positive control in this study is a HMG-CoA reductase inhibitor and since Johnston[20] demonstrated that the elevation of serum cholesterol levels following i.p. injection of poloxamer 407 solution to rats was due to stimulation of 3-hydroxy-3-methylglutaryl-Co-enzyme A (HMG-CoA) reductase activity in the liver by the poloxamer vehicle.

Flowers of the *P. granatum* contains different tannins such as ellagic acid and gallic acid,[27] 1, 2-di-O-galloyl-4, 6-O-(S)-hexahydroxydiphenyl β-D-glucopyranoside, pomegranatate[28] as well as different flavonoids such as apigenin, punicaflavone etc.[29] Besides, the flower also contains different triterpenoids: ursolic acid, maslinic acid and asiatic acid.[30] The observed antihyperlipidemic activity of the flower extract of *Punica granatum* may be due to the presence of the above mentioned flavonoids, tannins and triterpenoids as previously it has been reported that flavonoids[31], tannins[8] and terpenoids[32] possess antihyperlipidemic activity.

In conclusion, the present study demonstrated that the methanolic fraction of pet ether extract of flowers of *Punica granatum* possesses potential antihyperlipidemic acitivity. The results imply that the flower extracts of the plant may be used for the prevention and/or treatment of atherosclerosis which leads to different coronary artery diseases (CAD). Further investigations are required to elucidate the mechanism of action of the extracts.

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Future Position of *Crocus sativus* as a Valuable Medicinal Herb in Phytotherapy

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Sir,

Nowadays, there are numerous reports about the bioactivity and pharmacological effects of the plants as well as finding the active phytoconstituents.[1] In diverse countries, there is a great interest to treatment of several illnesses by traditional herbal medicine. This is because of the documented side effects and cost of synthetic drugs and not exactly on the basis of the higher safety or effectiveness of herbal medicine.[2]

Recently, some reports have been published about the clinical evaluation of the efficacy of saffron in mild to moderate depression.[3] The studies reported that saffron was more effective than placebo and at least equivalent to therapeutic doses of Imipramine and Fluoxetine and no significant differences were found in adverse effects in any of the studies. Unfortunately, clinical trial of saffron is restricted to the administration of dried stigma parts of the species *Crocus sativus*. Saffron is the object of frequent adulteration. Furthermore, the certification of the origin and quality of saffron as a medicinal food led to the frequently usages of chemical and molecular techniques.[4]

There has been another important point that saffron is generally not toxic when ingested in culinary amounts but a lethal dose at 20 g and an abortifacient dose at 10 g have been indicated in the literature. Adverse reactions such as rhino-conjunctivitis, bronchial asthma, cutaneous pruritus, and a case report of anaphylaxis have been existed.[5]

Based on the traditional usage of saffron as a spicy plant, a concern about the mentioned studies is the lack of adequate high-quality clinical studies by different group of scientists which is necessity to prove this effectiveness in other nationalities. Another question raised from those studies is how can treat the depressed patients by such an expensive natural medicine? However, *Crocus sativus* is the most expensive cultivated herb in the world and hence, saffron is the high price spice in the world market, which is due to the labor cultivation, harvesting, and handling. For this reason, the researches on this field should be directed to the methods in which higher amount of safranal (as the main effective compound) can be produced by chemical synthesis and/or biotechnological productions. Despite consuming many reports about the various pharmacological activities from this valuable medicinal herb[6], more well-designed clinical studies are suggested before administration in psychological conditions as well as the physicochemical studies on various populations of this valuable spicy plant to standardize the plant material.[7]

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