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Full Length Research Paper

Evaluation of antibacterial activity and modulatory effect of the hexane fraction from methanol extract of *Cordia verbenacea* DC leaves

Alves Erivania Ferreira*, Santos Beatriz Sousa, Coutinho Henrique Douglas Melo, Aguiar José Junior dos Santos, Costa José Galberto Martins and Matias Edinardo Fagner Ferreira

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Medicinal plants have been used since the beginning of civilization, and even today, it is still widely used mainly due to the lack of necessary information on their physical and chemical components. *Cordia verbenacea* DC is a typical plant of the Brazilian coast, popularly known as "grass whaling" and is mainly used as antimicrobial agents, anti-inflammatory and analgesic. This study was conducted in order to identify the phytochemical analysis of the main secondary metabolites, and also to evaluate the antibacterial effect and modulate bacterial resistance to aminoglycoside, the hexane fraction of the methanol extract of *C. verbenacea* (HFMECV) against the standards and resistant strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The phytochemical screening showed the presence of compounds such as tannins flobatenics, flavones, flavonols, xanthonenes, flavononols, leucoanthocyanidins, catechins, flavones and terpenes. Regarding antibacterial activity, the fraction showed minimum inhibitory concentration (MIC) $\geq 1024 \mu\text{g/ml}$, being a value not clinically relevant, but when combined with the sub-MIC, the aminoglycosides antibiotics showed synergism. With these results, we conclude that the HFMECV presented itself as an excellent modulator of bacterial resistance to aminoglycosides, thereby indicating a possibility that through further research there could arise a new herbal that work with modulating function and antibacterial.

Key words: Aminoglycosides, phytochemical analysis, antibacterial activity, minimum inhibitory concentration (MIC), modulation.

INTRODUCTION

Since the beginning of civilization, there is a great use of medicinal plants primarily as a therapeutic or for the prevention and cure of certain diseases. This practice is widely used today because the general population does not have the necessary information on chemical, physical

and constituents of many existing plants, as it is in the case of some exotic plants (Viegas et al., 2006; Veiga Junior, 2008).

A family of medicinal plant largely researched is the Boraginaceae, which have been found with about 100

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genera and over 2000 species distributed throughout the planet. A species well known today is *Cordia verbenacea* DC. it is a perennial and shrub located largely around the Brazilian coast and popularly known as "erva baleeira" (Sertié et al., 2005). *C. verbenacea* is widely used as anti-inflammatory and analgesic, and confirmed its anti-inflammatory effects through pharmacological tests (Rosa et al., 2008; Matias et al., 2010). Currently, medicinal plants are of great importance especially to the pharmaceutical industries, as these extracts have produced active compounds that may be part of the new formulations of synthetic drugs (Simões and Schenkel, 2002). This search for new components is often due to dissatisfaction with allopathic medicine for side effects through the inappropriate use of drugs (Rates, 2001; Ribeiro et al., 2005) or due to the growth and spread of microorganisms resistant to existing drugs (Penha et al., 2011).

Pseudomonas aeruginosa are a group of bacteria responsible for nosocomial infection in immune-compromised patients and they can be found in water, soil, plants and animals as well as in the normal intestinal flora of the human body (Brooks et al., 2009). *Escherichia coli* type bacteria are gram-negative participants in the human gastrointestinal flora, and they are the major cause of diarrheal and urinary tract diseases (Cunha et al., 2012). *Staphylococcus aureus* bacterial strains are also responsible for hospital infections, but also the leading cause of purulent infections of carbuncle and furuncle (Matias et al., 2010).

This study was conducted in order to identify the main classes of secondary metabolites by phytochemical, and also to evaluate the antibacterial effect of modulating bacterial resistance to aminoglycoside and the hexane fraction of the methanol extract of *Cordia verbenacea* (HFMECV) against bacterial strains and patterns of multiresistant.

MATERIALS AND METHODS

Bacterial material

The following bacterial strains used were: *S. aureus* (ATCC25923 and SA358), *E. coli* (ATCC10536 and EC27) and *P. aeruginosa* (ATCC15442 and PA03). All strains were maintained in Heart Infusion Agar (HIA) at 37°C.

Plant material

Leaves of *C. verbenacea* DC. were collected in the city of Crato, Ceará, Brazil. The plant material was identified and a voucher specimen was deposited in the Herbarium Prisco Bezerra of Federal University of Ceará - UFC, under No. 044 171.

Preparation of the extract, fraction and antibacterial test solution

For preparation of extracts, leaves were collected and then passed

through the grinding process which increased its contact surface, the grinded leaves were wrapped in a container with solvent (methanol) volume enough to submerge the whole plant material, remaining there for 72h. After this period, the eluent was filtered through filter paper to separate the solid concentrated in rotary vacuum condenser (model Q-344B - Quimis, Brazil) (model Q-214M2 - Quimis, Brazil). The extracts mixed with silica were then subjected to vacuum filtration process with solvents of different polarity. For microdilution tests, solutions prepared from the fraction in a concentration of 10 mg/ml, dissolved in DMSO (dimethyl sulfoxide) then diluted with distilled water to a concentration of 1024 µg/ml were used. Preliminary evaluation were performed with DMSO and showed no toxicity and interference with concentration below 5%. DMSO used at concentration of 9%, during the tests ranged 4.5 to 0.03%, and is considered non-toxic concentration.

Phytochemical screening

Phytochemicals tests were performed to detect the presence of glycosides, saponins, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids using the method described by Matos (1997). The tests are based on visual observation of color change or precipitate formation after addition of specific reagents.

Antibacterial activity and modulating resistance to aminoglycosides

The MIC was determined from microdilution broth, using an inoculum of each strain of 100 µL/ml suspended in Brain Heart Infusion (BHI liquid) to give a final concentration of 10⁵ CFU/ml. In microdilution, plates with 96 wells were distributed into each well, promoting 1:1 dilution series of the test solution, promoting concentrations ranging from 512 to 8µl. To obtain the controls, antibiotics: amikacin, gentamicin and neomycin were used. The plates were all incubated for 16 to 24h at 37°C. The MIC was determined as the lowest concentration able to inhibit bacterial growth which was performed by triplicate. To evaluate the modulating action of bacterial resistance towards antibiotics, microdilution methodology described earlier was used. The modulatory activity determined by the MIC of aminoglycosides, where all the plates were incubated for 16 to 24h at 37°C. Results were obtained by triplicate, tabulated from the average geometrical data, statistically analyzed by two-way ANOVA and subjected to Bonferroni post-test in GraphPad Prism 5.0 (Matias et al., 2013).

RESULTS

The phytochemical screening was performed to identify the classes of secondary metabolites. Table 1 presented the presence of several bioactive compounds in the extract fraction rated as: tannins, flavonoids, flavonols, xanthenes, flavonols, leucoanthocyanidins, catechins, flavanones and terpenes. In trials to test the antimicrobial activity of the hexane fraction of the methanol extract of *C. verbenacea* (HFMECV), for all bacterial strains tested (ATCC and multiresistant Gram positive and negative) was observed MIC value ≥ 1024 µg/ml. Being the amount considered clinically significant when the MIC is MIC ≤ 256 µg/ml. Figure 1 shows the interference of HFMECV on the activity of

Table 1. Phytochemistry screening of hexane fraction of the methanol extract *C. verbenacea* DC. leaves.

Natural product	Phenols	Tannins pirogalics	Tanins flobatenics	Anthocyanins	Anthocyanidins	Flavones	Flavonols	Xanthones	Chalcones	Aurones	Flavononols	Leuco-anthocyanidins	Catechins	Flavonones	Alkaloids	Terpenes
HFMECV	-	-	+	-	-	+	+	+	-	-	+	+	+	+	-	+

(+) Presence, (-) absence. HFMECV (hexane fraction of the methanol extract of *C. verbenacea* DC).

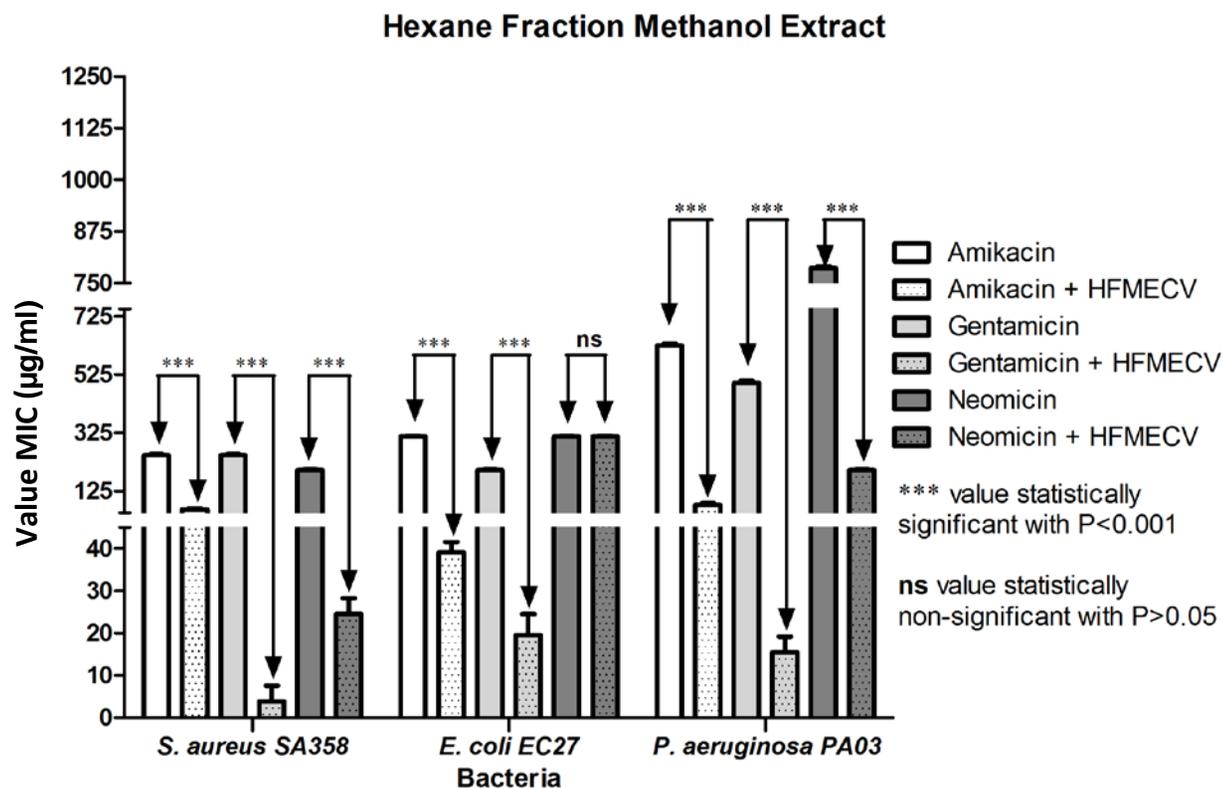


Figure 1. Activity modulating bacterial resistance against the aminoglycosides by hexane fraction of the methanol extract of *Cordia verbenacea* DC. leaves (HFMECV).

aminoglycosides (gentamicin, amikacin and neomycin) showing that there was a reduction in

antibiotic concentration when combined with HFMECV sub-inhibitory concentration in bacterial

strains tested. All other antibiotics tested against resistant strains were significantly relevant except

opposite strain of *E. coli* which showed no statistical significance in testing the modulation with the aminoglycoside neomycin.

DISCUSSION

Plants tend to synthesize some products or sub-products that have various applications such as attractive to pollinators and protective function. One of such product that serves as protection against fungi and bacteria are the flavonoids, where currently there are over 6000 types that have been described in the literature, among them are: flavonols, flavones, flavanones and catechins which are constituents of *C. verbenacea*. Flavonoids have certain capabilities, among them are antimicrobial and antiviral which are the most important (Machado et al., 2008) and so may be an explanation for the tests modulation minimum concentrations observed when considering only the use of antibiotics used.

Many medicinal plants used by the general population in their compositions have a class of metabolite known as tannins which have antibacterial properties and they mainly cause a higher sensitivity in *S. aureus* strains, but also may sensitize strains of *Streptococcus pneumoniae*, *Bacillus anthracis*, and *Shigella dysentery* (Monteiro et al., 2005). Combinations of antibiotics of the aminoglycoside type with hexane fraction of methanol extract have synergistic actions when tested with the bacterial strains multiresistant patterns and promoting the reduction of MICs, thus leading to a decreased concentration of antibiotics in order to obtain a therapeutic effect (Figueredo et al., 2013).

This strategy is known as "herbal shotgun" or "the synergistic multi-target effect" because the components obtained from these compounds are medicinal plants, most often by multi combined extracts to be responsible for acting not only on one site action, but also on a variety of targets that may have a synergistic or antagonistic action depending on its composition. This can occur not only with combinations of more extracts but also with the combination of antibiotics with natural synthetic (Matias et al., 2013).

The most favorable results were opposite lineage *S. aureus* possibly because it is a bacterium that has gram positive cell wall. The other strains tested which include *E. coli* and *P. aeruginosa* results were not as relevant as *S. aureus*, but this may have occurred because these bacteria have a greater chemical complexity (Rodrigues et al., 2012).

CONCLUSION

Conclusively, it was found that the hexane fraction of the methanol extract has no clinical significance since it has relevant MIC ≥ 1024 $\mu\text{g/ml}$, but has a higher efficiency when used together to aminoglycoside antibiotics. There-

fore, new research is suggested to have more information on the mechanisms of action and possible development of herbal medicines with antibacterial activity, and in modulating the products obtained from the leaves of *C. verbenacea*.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Hepatoprotective medicinal plants used by the Gond and Bhill tribals of District Raisen Madhya Pradesh, India

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The ethnobotanical survey of the medicinal plants used in the management of jaundice (hepatic disorder) was carried out in the District Raisen of Madhya Pradesh. The herbalists, herb sellers and traditionalists were interviewed by the administration of questionnaires. Floristically, the area is placed in Malwa plateau region of Madhya Pradesh. Aims of the study were to document the medicinal plant resources and their use patterns by the tribal people. A total of 19 plant species belonging to 16 families were reported as locally used for the hepatic disorder purposes. Majority of the recipes are prepared in the form of decoction from freshly collected plant parts. Mostly a single species is used and taken orally. Field observations showed that vegetation of the area was generally threatened with the ignorance of local communities. The trends like urbanization, deforestation, over grazing, habitat fragmentation, unscientific extraction of natural vegetation, introduction of the exotic taxa and habitat loss were the visible threats. Measures for the conservation of plant resources especially medicinal plants of Raisen district of Madhya Pradesh are urgently needed. Some of the important species for the alleviation of hepatic disorders are *Aegle marmelos*, *Azadirachta indica*, *Cajanus cajan*, *Cuscuta reflexa*, *Gloriosa superba* and *Ricinus communis*.

Key words: Jaundice, tribe, medicinal plant, conservation, hepatic disorders.

INTRODUCTION

Jaundice is the most common of all liver disorders. It is not a disease but rather a sign that can occur in many different diseases. Jaundice is the yellowish staining of the skin and sclerae (the whites of the eyes) that is caused by high levels in blood of the chemical bilirubin. The color of the skin and sclerae vary depending on the

level of bilirubin. It is a condition in which yellow discoloration of the skin and mucous membranes occur due to an increase in the bile pigments, namely, bilirubin in the blood. When the bilirubin level is mildly elevated, they are yellowish. When the bilirubin level is high, they tend to be brown. Jaundice is caused by bilirubin which

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comes from red blood cells. When red blood cells get old, they are destroyed (Balakrishnan et al., 2011). Hemoglobin, the iron-containing chemical in red blood cells that carries oxygen, is released from the destroyed red blood cells after the iron it contains is removed. Jaundice may be caused by an obstruction of the bile ducts which normally discharge bile salts and pigment into the intestine. The bile gets mixed with blood and this gives a yellow pigmentation to the skin. The obstruction of the bile ducts could be due to gallstones or inflammation of the liver, which is known as hepatitis, and is caused by a virus. Jaundice may result from various diseases or conditions that affect the liver, like hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatitis E, autoimmune hepatitis, liver cirrhosis, liver cancer, hemolytic anaemia and malaria. There is no unique treatment for jaundice (hepatitis) by prescribing modern allopathic and homeopathic medicine (Agarwal, 2001; Goel and Bhattacharya, 1981).

Jaundice indicates excessive levels of conjugated or unconjugated bilirubin in the blood and is clinically apparent when the bilirubin level exceeds 2 mg/dl (34.2 μ mol/L). It is most apparent in natural sunlight. In fact, it may be undetectable in artificial or poor light. In fair-skinned patients, jaundice is most noticeable on the face, trunk and sclerae; in dark-skinned patients, it is noticeable on the hard palate, sclerae, and conjunctivae. Pseudo jaundice may be found in black patients with pigmented sclera, from carotinemia, uremia (a sallow yellowish pallor), and quinacrine (a yellow-green color). Causes of jaundice can be classified into pre-hepatic, hepatic or post hepatic (Saleem et al., 2008; Stickel and Schuppan, 2007; Chang et al., 2008).

In this paper, our focus is on post hepatic causes of jaundice (obstructive or surgical cholestasis) as this is more relevant to surgeons. Obstructive jaundice is not a definitive diagnosis and early evaluation to establish the etiology of the cholestasis is crucial to avoid secondary pathological changes (e.g. secondary biliary cirrhosis) if obstruction is not relieved.

In this context, the present study is the first milestone with particular emphasis on antiviral application of medicinal plants for jaundice. Chemical that remains in the blood after the iron is removed becomes bilirubin. The symptoms of jaundice are extreme weakness, headache, fever, loss of appetite, severe constipation, nausea and yellow discoloration of the eyes, tongue, skin and urine. The patient may also feel a dull pain in the liver region. Obstructive jaundice may be associated with intense itching. Pulse, tongue, nail and eye examinations are important diagnostic methods used to reveal a person's body humour and its imbalance. This will help the doctor in treating the disease. Quite a handful of tribes reside in every nook corners of Raisen district of the state Madhya Pradesh. The tribal (Figure 1) community via Gond, Bhils, Pardhan, Agariya, Ojha, Nagarchi and Solhas are one of the nomadic tribes who have settled down in villages. The historical evidences reveal that they associated

themselves with the forest which provides them all their day-to-day requirements. The main objective of this paper is to analyze how these tribal pastoralists and peasants agriculturists have interacted with the forest resources in utilizing them for jaundice. The tribals of the district are still using the natural resources available in their surroundings to treat many diseases and accidental derangements. They believe in mantras and tantras also, in the view of snake bites, they are using the old tradition of treatment, that is, by mantras along with the administration of particular plant drugs.

Study area

Raisen District of the Bhopal commissioners division lies in the central part of Madhya Pradesh. The District is situated between the latitude 22° 47' and 23° 33' North and the longitude 77° 21' and 78° 49' East. It lies mostly on the Malwa Plateau and partly in the Narmada Valley. The District has an irregular shape. The Tropic of Cancer passes through the Northern part of the District. It is bounded in the West by Sehore District, in the North by Vidisha District, in the East and North-East by Sagar District, in the South-East by Narsimhapur District, and in the South by Hoshangabad and Sehore Districts. The Narmada river flows along the South-Eastern boundary of the Districts and separates it from Narsimhapur and Hoshangabad District (Figure 2). The total area of the District is 8,395 sq. km which contains 1.93% of the states total area. The District Raisen has a dry climate except in the Southwest monsoon season. The year may be divided into four seasons. The period from March to about the second week of June is the hot season. The South-West monsoon season which follows thereafter continues up to end of September. October and November constitute the post-monsoon season. The cold season is from December to the end of the February. The temperature obtained in the area is mild for the latitude due to the effect of altitude. Thus, the climatic conditions in the District are normal. During the summer season, the mercury raises up to 42°C and during the winter, the climate is cold and the temperature is around 5°C. The average annual rainfall in the District is 1312.6 mm. (50.693 inches). The region around Bareilly and Sultanpur gets the lowest rainfall in the District and that around Chiklod gets the highest rainfall. About 22% of the annual rainfall in the District is received during the South-West monsoon months from June to September, July being the rainiest month.

MATERIALS AND METHODS

In the present investigation we focused on medicinal plants used to treat jaundice and hepatitis. The study was carried out by interviewing respondents in seventeen remote sites. Intensive field work has been undertaken for a period of three years (2008 to 2011), covering different seasons so as to gather information on



Figure 1. Tribal community.



Figure 2. Map showing Raisen District.

each of the plant species found to be used in traditional healing practices of Raisen tribes of Madhya Pradesh of India. A total of 95 informants, including 45 female, 40 male and 10 traditional healers were interviewed. Information was gathered by taking interview of local herbalists using structured questionnaires in some cases and documentation of verbal information and personal observations. Herbalists were selected on the report of local informants. Before the interview, the respondent was explained with the aim of the study, followed by verbal consent. Each of the healers was selected based on their previous experience of using medicinal plants in treatment and the data obtained from one healer was cross verified with the other. The vernacular name, mode of preparation and also disease treated were recorded. In certain cases, where the healers did not know the name of the disease, the names of the diseases were given on the basis of symptoms described by them. The collected specimens were tagged and herbarium sheets were prepared for each of the species. The specimens were identified consulting Flora of British India (Hooker, 1897), Flora of Bhopal (Oommachan, 1976) and Flora of Marathwada (Naik, 1998). Some of the noteworthy contributions in the field of ethnobotany of the centrally located state of the country encompass the work of Jain (1963, 1964, 1987), Khan and Chaghtai (1979, 1981), Khan et al. (1981, 1984, 1992), Ahmad et al. (2010), Khan and Zaheer (1981) and Ahirwar (2010). As is evident from these references, there is

very little ethnomedicinal information available for the district Raisen. The information recorded in the field was further compared with the works of Jain and Singh (1994), Judah and Oommachan (1994) and Masih (1997).

Recently, Srivastava (2011) has explored the medicinal plants used by tribals of Bandhavgarh National park of Madhya Pradesh, and Ahirwar and Singh (2011) have reported some anti diabetic plants from Dindori district of Madhya Pradesh. As said earlier, the studies is pertaining to floristics and ethnobotany, so far as the state of the Madhya Pradesh is concerned and perusal of literature reviews that the district Raisen is almost unexplored from this point of view. A perusal of literature reveals that only the preliminary study of Goharganj of this district is carried out by Khan and Haque (1981). Plants were collected in flowering and fruiting conditions and confirmed by using different herbaria. Specimens were dried, pressed, poisoned and mounted on herbarium sheets. All collected specimens were identified with the help of available literature. Finally, specimen identification was authenticated consulting Saifia Science College, Herbarium, Barkatullah University, Bhopal, India. Set of herbarium sheets were deposited in the herbarium for future reference. The alphabetic arrangement of all the plant species were made along with information on vernacular names, place of collection, parts used, mode of uses and disease classification.

Table 1. Medicinal plants used by the tribals of Raisen for the management of Jaundice.

S/No	Botanical name/family/local name/ voucher specimen number	Part used	Chemical constituents	Mode of administration
1	<i>Achyranthes aspera</i> Linn, Amaranthaceae, Latzeera (Patajada), ZAL139	Root	Alkaloids, glycosides, saponins, and dihydroxyketone	The fresh roots (5 g) are ground to fine powder given to patient twice a day for about one week to cure the patient
2	<i>Aegle marmelos</i> Correa ex Koen., Rutaceae, Beel/Bel, ZAL213	Leaf	Pyridine, scopoletin, maresin	The fresh leaves (10 ml) are extracted and its juice mixed with five pieces of black pepper. Two spoons of this juice given twice a day for 10 to 15 day cures the patient
3	<i>Azadirachta indica</i> A. Juss., Meliaceae, Neem, ZAL225	Leaf, stem bark, fruit and flower	Azadirachtin, melianine A, B, Azadirone, melianone, nimbin, nimbidin nimbidinin, vilasinin and melicitrin	Mixture of same quantity of leaf powder, fruit powder, stem bark powder and flower powder, taken one spoonful with one spoonful ghee and honey (½ spoon) twice a day for ten days
4	<i>Cajanus cajan</i> (L.) Mill Sp., Fabaceae, Arhar, Tuar, ZAL202	Leave	Coumarin cajanuslactone, stilbenes, cajanin	The juice of leaves is mixed with black pepper and butter milk. Two spoonful of this mixture is given to patient thrice a day for two weeks cures the patient
5	<i>Capparis zeylanica</i> Linn, Capparidaceae, Endriand (Ardadanda), ZAL136	Fruit	Thioglucosides and glucocapparin	The fruits are washed and seeds are taken out from the fruit then seeds are dried for 2 to 3 h. Then (100 to 200) seeds are fried in cows ghee for one minute. After it 2 or 3 seeds are taken daily for 7 to 8 days in empty stomach with water
6	<i>Cosmos sulphureus</i> Cav, Asteraceae, Jungli gaindha (Peela gaindha), ZAL253	Fruit	Quercetine	The fresh fruits are crushed mixed with water and are prepared in the form of tablets given to the patient twice a day for about one month to treat jaundice
7	<i>Cuscuta reflexa</i> Roxb., Convolvulaceae, Amarbel(Akasbel), ZAL182	Stem	Alcohol, systolic and tachyphylaxis	The fresh small pieces of the stem (10 to 12) are given to the patient twice a day for about twenty days to recover from the jaundice
8	<i>Eclipta alba</i> (Linn.) Hassk., Asteraceae, Bhrigraj (Babri), ZAL252	Leave	Polyacetylenic thiophenes	One glassful of leaves decoction is taken twice a day for two weeks to relieve jaundice
9	<i>Gloriosa superba</i> L., Liliaceae, Ladael (Languli), ZAL237	Tuber	Colchicine, lumicolchicines, demethylcolchicine, luteolin	Garland of fresh tuber pieces put around the neck of patient for 10 to 15 days to treat the patient
10	<i>Holoptelea integrifolia</i> Planch, Ulmaceae, Churale (Chilbil), ZAL119	Leaf	Hexacosanol and octacosanol	The decoction of the leaf is used for bath after applying the ash of the <i>Achyranthus aspera</i> on the body cures the jaundice patients
11	<i>Lawsonia inermis</i> L., Lythraceae, Mehnidi, ZAL184	Leave	Xanthones and Laxanthones	The leaves one gram are crushed mixed with 4 black peppers and made into a paste and taken with two glass of milk once a day for 15 days to cure jaundice

Table 1. Cont'd

12	<i>Madhuca indica</i> J.F. Gmel., Sapotaceae, Mahwa, ZAL221	Stem bark	Saponin and bassianin	Decoction of stem bark is used for bath after applying the ash of <i>Achyranthes aspera</i> on body once a day for 3 days
13	<i>Morus nigra</i> Linn., Moraceae, Shatoot, ZAL201	Fruit	Phenols and alkaloids	The decoction of the young fruits is taken. One cup with one teaspoonful of sugar is given twice a day before meals for about one week
14	<i>Phyllanthus niruri</i> Linn., Euphorbiaceae, Jaaliriya (Jungli amla), ZAL128	Whole plant	Flavonoids, tannins, alkaloids and sterol	The dried plants (2 to 3) are crushed into a fine powder and put into water or milk and half glass of it is given to the patient for (3 to 5) days once a day in the empty stomach for the ailment of jaundice
15	<i>Phyllanthus urinaria</i> L., Euphorbiaceae, Boine awla (Lal- bhuin anvalah), ZAL116	Leave	Alkaloids, tannins, corilagin, rutin etc	The fresh leaves are ground to the fine powder, and put it into milk with the addition of water. The (6 g) of it is given to the patient once a day for about three days
16	<i>Psidium guajava</i> Linn., Myrtaceae, Amrood, ZAL240	Leave	Beta-sitosterol, Uvaol, oleanolic acid and ursolic acid etc	Three cooked leaves in oil are taken twice a day for 3 week
17	<i>Ricinus communis</i> Linn., Euphorbiaceae, Andi (Arandi), ZAL173	Leave	Lipids and phosphatids	The decoction of the leaves is taken one glassful twice a day for about 2 to 3 weeks
18	<i>Solanum nigrum</i> L., Solanaceae, Makoo, ZAL147	Stem Bark	Pinosresinol, syringaresinol, medioresinol, scopoletin etc	The fresh decoction of the stem bark and leaves is given to the patient twice a day for about one week to relief the patient against the jaundice.
19	<i>Tribulus terrestris</i> Linn., Zygophyllaceae, Choti Gokhru, ZAL231	Whole Plant	Steriodal saponins etc	The plant is made into paste along with equal quantity of whole plant of <i>Amaranthus tricolor</i> . 2 spoonfuls of paste is mixed with cow milk and given on empty stomach for about one week to cure the jaundice

RESULTS AND DISCUSSION

Data of jaundice plants investigation is compiled in (Table 1) and the plants species are arranged in alphabetic order. A total of 19 plant species belonging to 16 families have been reported for the treatment of jaundice. For each plant species, botanical name, family, local name, parts used, chemical constituents, voucher specimen number, preparation and application are provided.

The most dominating families were

Euphorbiaceae with three species, Asteraceae with two species, followed by Amaranthaceae, Convolvulaceae, Fabaceae, Meliaceae, Liliaceae, Lythraceae, Rutaceae, Sapotaceae, Ulmaceae, Moraceae, Myrtaceae, Solanaceae, Zygophyllaceae and Cappridaceae with one species each. Some of the highly utilized plant species include, *Achyranthes aspera*, *Aegle marmelos*, *Azadirachta indica*, *Cajanus cajan*, *Capparis zeylanica*, *Cosmos sulphureus*, *Cuscuta reflexa*, *Eclipta alba*, *Gloriosa superba*, *Holoptelea*

integrifolia, *Lawsonia inermis*, *Madhuca indica*, *Morus nigra*, *Phyllanthus niruri*, *Phyllanthus urinaria*, *Psidium guajava*, *Ricinus communis*, *Solanum nigrum* and *Tribulus terrestris* (Table 1).

Different plant parts were used to cure jaundice. Among these fruits, whole plants were highly utilized followed by root, leaves, seeds, bark and rhizome in decreasing order (Table 1). Data presented in Table 1 shows that thirty five medications were used for jaundice that can be divided into two categories: those that are

prepared from (i) single plant and (ii) from more than one plant species. In majority of the cases, these medications were prepared by using water as a medium and administered along with buttermilk, water and sugar. In all the cases, mode of application was oral. In regard to the patients' condition, the preparations were use more than two times daily from a week to month till the problem is cured.

Jaundice results from various diseases or conditions that affect the liver. Mostly, it is due to viral hepatitis A, B, C, D and E, liver cirrhosis and liver cancer. Some of the plant species mentioned in the present study used to cure jaundice have been investigated for their antimicrobial activities (Pal et al., 2006). For example, the hexane and alcoholic extracts of *Phyllanthus emblica* (fruit), *Tamarindus indica* (fruit) and *Punica granatum* (fruit - pericarp) were found to be antimicrobial while *Morus alba* (fruit) did not show antimicrobial activity (Ahmad et al., 1998). Aqueous extract of *Tamarindus indica* (fruit) shows positive response against antimicrobial activity. By comparing these plant species recorded to cure jaundice with available pharmacological literature reported from other regions of the subcontinent and world, it appears that there are many medicinal plant species in the area that were not reported in other locations. To our knowledge, the use *P. emblica*, *P. granatum* to cure jaundice, have never been reported before. Hepato-protective effect of *Aegle marmelos* on rats was reported by Vinodhini (2007). Decoction of fresh plant material of *Boerhaavia procumbens* is used for the said purpose in the study areas, while other authors (Shah and Khan, 2006; Katewa et al., 2004; Sing et al., 2002; Khan et al., 2000) reported that leaves and roots of this plant are used for jaundice, swelling, watering of eyes, anaemia, asthma, dropsy, gonorrhoea, stomach disorders, sore throat, to relief pain, typhoid, as cooling, antispasmodic and astringent. Dried fruit powder of *P. emblica* is used for said purpose in the study areas, while Ahmed et al. (2007) and Shinwari and Khan (1998) reported that fruit, leaves and bark of this plant are used as eye tonic, astringent, cooling, diuretic, laxative, refrigerant, aperients, for asthma, diarrhea, dysentery, cold and cholera.

Conclusion

Medicinal plants play a vital role in the life by serving good health and well being of mankind. Present study reveals unique utilization of medicinal plants by the tribes belonging to Raisen district of Madhya Pradesh. In the present investigation, 19 medicinal plant species used to treat jaundice were reported and documented. The use of these plants to treat various illnesses is still needed by the communities, because of poor socio-economic conditions the high cost and a difficult access to allopathic medicines. The majority of the reported species

are wild and rare. These demand an urgent attention to conserve such vital resources so as to optimize their use in the primary health care system. Nowadays, conservation of traditional knowledge is greatly menaced by a lot of factors related to modernization of the region and lack of interest in traditional healers, in transferring it to next generation. It is therefore, a need of the hour to save the cultural heritage of the natives, by confirming the therapeutically used plants with scientific criteria. In this context, screening for active substances and testing their activities against jaundice and hepatitis causing organisms form an interesting subject for the future studies.

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Conflict of interest

We declare that none of us has any conflict of interest.

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Full Length Research Paper

Anti-inflammatory effects of *Portulaca oleracea* L. on the LPS-induced RAW 264.7 cells

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Portulaca oleracea L. (Portulacaceae) has been widely used as a folk medicine in many countries. The present study investigated the effects of aqueous extract of *P. oleracea* (PO) on pro-inflammatory mediators secreted from lipopolysaccharide (LPS) - activated macrophage cells (RAW 264.7) as an established inflammation model. The reference drug indomethacin was used for comparison purpose. PO did not show cytotoxic effects at the concentrations tested. When RAW 264.7 macrophages were treated with PO together with LPS, a significant concentration, dependent inhibition of nitrogen oxide (NO) production was detected. Western blotting revealed that PO blocked protein expression of iNOS in LPS - stimulated RAW 264.7 macrophages, significantly. The change in the contents of PGE₂, IL-6 and TNF- α were monitored by enzyme linked immunosorbent assay (ELISA). Compared with indomethacin, PO has much more potency and inhibited the production of PGE₂, IL-6 and TNF- α in LPS- induced RAW 264.7 cells at concentrations of 0.05, 0.1, and 0.2 mg/ml ($p < 0.05$). These results suggested that PO might have a potential therapeutic effect by inhibiting the inflammation process such as arthritis.

Key words: *Portulaca oleracea*, inflammation, extract, therapeutic effect.

INTRODUCTION

Inflammation preserves the host against tissue injury and microbial invasion. An inflammatory reaction is self-limiting and includes the decrease of pro-inflammatory protein expression and increased reaction of anti-inflammatory proteins that promote the innate immune responses (Murakami et al., 2007). Drugs had been prescribed as steroidal anti-inflammatory drugs (SAID)

and non-steroidal anti-inflammatory drugs (NSAID) to treat acute inflammatory diseases, but these ordinary drugs have not been successful against chronic inflammatory diseases such as rheumatoid arthritis (RA), and atopic dermatitis (AD). Anti-inflammatory activity has been studied by the activity of inflammatory mediators and pro-inflammatory cytokines in the inflammation of

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RAW 264.7 cell (Hong et al., 2012). Macrophages are fundamental cells in cell-mediated innate immune reaction with reduced functions and the capacity to start acute inflammatory response (Gordon, 2007). The stimulation of macrophages with lipopolysaccharide (LPS) results in a number of functional reaction production of nitric oxide (NO) and cytokines increased (Olszanecki et al., 2002).

During cell signaling, materials such as NO, cytokine, growth factor, PGE₂ and other inflammatory mediators are precise subjects in immunologic and inflammatory study. IL-1 β , IL-6 and TNF- α are known to make fever, inflammation, and tissue destruction (Dinarello, 2000). However, overproduction of these inflammatory mediators leads to many diseases such as rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis. Indomethacin is used to relieve pain and inflammation in a wide range of musculoskeletal conditions, including various forms of arthritis and gout. Indomethacin blocks the production of prostaglandins and is therefore effective in reducing inflammation and pain (Shakeel et al., 2010).

Portulaca oleracea (PO) is from the Portulacaceae family, which has the highest rates of omega-3 fatty acids and anti-oxidant vitamins (Rahdari et al., 2012). PO has pharmacological effects including antibacterial (Leite et al., 2007), analgesic (Terra et al., 2007), skeletal muscle-relaxant (Chang et al., 2006) and wound-healing (Clancy et al., 1998). PO has high resistance to environmental stress such as drought and it is used as a medical plant in low-rainy regions (Rahdari et al., 2012). Most of the biologically active compounds associated with the referred multi-pharmacological effects of PO have not been shown thus far. The present study investigates the inhibitory effects of PO water extract on the production of pro-inflammatory mediators including NO, iNOS, PGE₂, IL-6 and TNF- α in LPS - induced RAW 264.7 macrophages.

MATERIALS AND METHODS

Macrophage RAW 264.7 cells were purchased from Amerocal Type Culture Collection (ATCC, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Gibco (Invitrogen, USA). Lipopolysaccharide (LPS, from *Escherichia coli* 0111: B4) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (USA). Param assay kit was purchased from R&D systems (Minneapolis, USA). The antibodies (Abs) used in this study included: anti-iNOS mAb, anti- β -actin mAb and anti-IgG-HRP, rabbit polyclonal Ab were purchased from Santa Cruz (USA).

Sources of plant materials

Total aerial parts of PO was purchased from Kyung Dong Market in Seoul, Republic of Korea, and then specimens were taxonomically identified by an oriental doctor, S.W. Lee at the National Institute of

Horticultural and Herbal Science, RDA. The voucher specimen (HPR-209) was deposited at the herbarium of Herbal Crop Research Institute (Eumsung, Republic of Korea).

Preparation of plant extracts

PO has been extracted by water traditionally in oriental medicine, PO (100 g) was extracted with 4 L of boiling water for 2 h, filtered and then lyophilized (25%, wt/wt). The powdered extract was dissolved in saline and then filtered through a 0.22 μ m syringe filter.

Cell culture

Murine macrophage RAW 264.7 cells (ATCC) were cultured at 37°C in Dulbecco's modified eagle's medium containing 10% fetal bovine serum, 2 mM glutamate, 100 unit/ml of penicillin and 100 μ g/ml of streptomycin in a humidified incubator with 5% CO₂. Cells were incubated with 1 μ g/ml LPS along with various concentrations of plant extracts for 24 h as indicated.

Protein isolation and western blot analysis

Raw 264.7 cells were stimulated in the presence of LPS (1 μ g/ml) with and without PO and indomethacin for 10 h. Protein (80 μ g) was loaded onto polyacrylamide gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Criterion system (Bio-Rad) at a constant voltage of 90 V. Proteins were subsequently transferred to poly(vinylidene fluoride) (PVDF) membrane (Millipore, Billerica, MA, USA) at a constant voltage of 15 V for 30 min and identified by using relative antibodies.

Anti-inflammatory activity

To investigate NO formation, nitrite (NO₂⁻) is measured since it is a stable and nonvolatile breakdown product of NO. The determination of nitrite relies on a diazotization reaction with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). Therefore, the nitrite accumulated in culture medium is a direct indicator of NO production (Green et al., 1982). A 100 μ l aliquot of each supernatant from the 96-well plate was mixed with 100 μ l Griess reagent and incubated at room temperature for 15 min. The concentration of total nitrite was determined by reading the absorbance at 550 nm and then calculated by a NaNO₂ dilution standard curve. The culture medium was collected after LPS treatment for PGE₂, TNF- α and IL-6 assays, respectively. The time points for PGE₂, TNF- α and IL-6 level were determined by a time course assay (data not shown). Levels of PGE₂, TNF- α and IL-6 in the culture media were determined with commercial enzyme linked immunosorbent assay (ELISA) assay kits (Park et al., 2004).

RESULTS AND DISCUSSION

Cell viability was tested throughout the experiments using MTT assay. At concentrations up to 0.2 mg/ml, no significant cytotoxicity was observed for any of the extract (data not shown). PO produced a dose-dependent inhibition of LPS-induced NO production (Figure 1A).

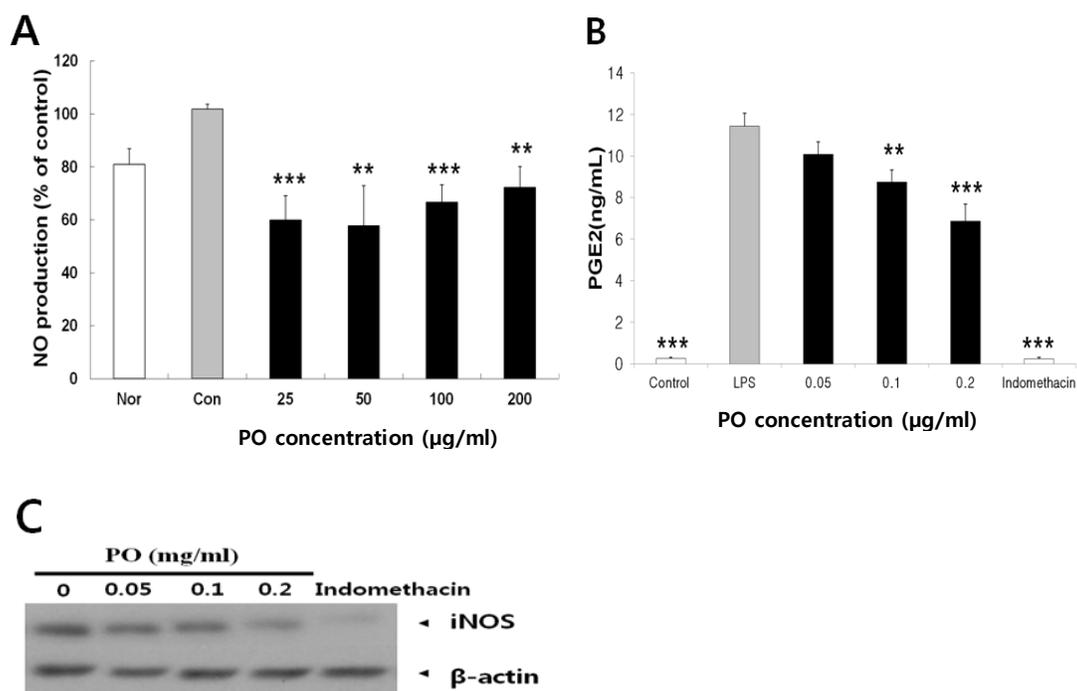


Figure 1. Effects of PO on NO, PGE₂ and iNOS in LPS-stimulated RAW 264.7 macrophages. (A) The normal group (Nor) was treated with media only. The control group (Con) was treated by the Griess reaction assay and expressed as a percentage of the control group. B. RAW 246.7 cells were treated with LPS (1 µg/ml) without and with PO (0.05, 0.1, and 0.2 mg/ml), and indomethacin (0.25 mM) for 12hrs prior to PGE₂ concentration being measured. C. Cells were incubated with of PO (0.05, 0.1 and 0.2 mg/ml), and indomethacin (0.25 mM) in the presence of LPS (1 µg/ml) for 10 hrs prior to analyzing iNOS protein (140 kDa) expression in RAW246.7 cells. Values are the mean±SEM of the three independent experiments. *** $p < 0.001$, ** $p < 0.01$ compared with LPS-stimulated group.

0.25 mM of indomethacin was used as a positive control in further studies to allow a comparison with PO anti-inflammatory activity and has often been used as a iNOS gene inhibitor (Terra et al., 2007). Figure 1A shows that the amount of NO released were 57.8 ± 3.3 , 55.6 ± 3.9 , 49 ± 6.1 , 43.6 ± 8.2 and $0 \mu\text{M}$ for 1, 5, 25, 125 and $625 \mu\text{g/ml}$, respectively. Whereas, LPS-treated cells produced a large amount of NO. PO IC₅₀ was $380 \pm 20.3 \mu\text{g/ml}$. Distinctly, the effect on NO inhibition indicates that PO has a great potency against inflammation. During the inflammatory process, large amounts of nitric oxide and PGE₂ are released by a wide variety of tissues and cells. LPS stimulation significantly increased PGE₂ production. An abnormal level of PGE₂ via COX activity is known to mediate inflammation. The results showed that the inhibition of PGE₂ synthesis were 10.1 ± 0.6 , 8.8 ± 0.6 and $6.9 \pm 0.8 \text{ ng/ml}$, for 0.05, 0.1 and 0.2 mg/ml, respectively (Figure 1B). The induction of PGE₂ was decreased by dose dependent of PO but can be mostly abolished by 0.25 mM of indomethacin.

PO was less potent than indomethacin in the terms of

inhibiting PGE₂. The expression of iNOS and the release of NO by macrophages are esteemed to play a significant action in the pathogenesis of various inflammatory diseases. LPS is the major component of endotoxin, arrests macrophage proliferation and activation of pro-inflammatory factors (Morrison and Ryan, 1987). Raw 264.7 cells were incubated for 12 h with LPS (1 µg/ml) in the absence or presence of PO (0.05 to 0.2 mg/ml). When the effect of PO on iNOS protein was examined in LPS-induced cells by Western blotting, the PO inhibited LPS-induced protein expression level of iNOS in a dose-dependent manner as shown in Figure 1C. PO inhibited iNOS expression in similar way as shown in LPS-induced nitric oxide synthesis. Therefore, the inhibition of iNOS expression may establish an effective new therapeutic agent for the medicine of inflammation and the prevention of inflammatory disease. A number of iNOS inhibitors have been known to reduce the production of inflammatory cytokines such as IL-6 and TNF-α. The mechanism where IL-6 and TNF-α act in concert to stimulate prostaglandin production, is however not well

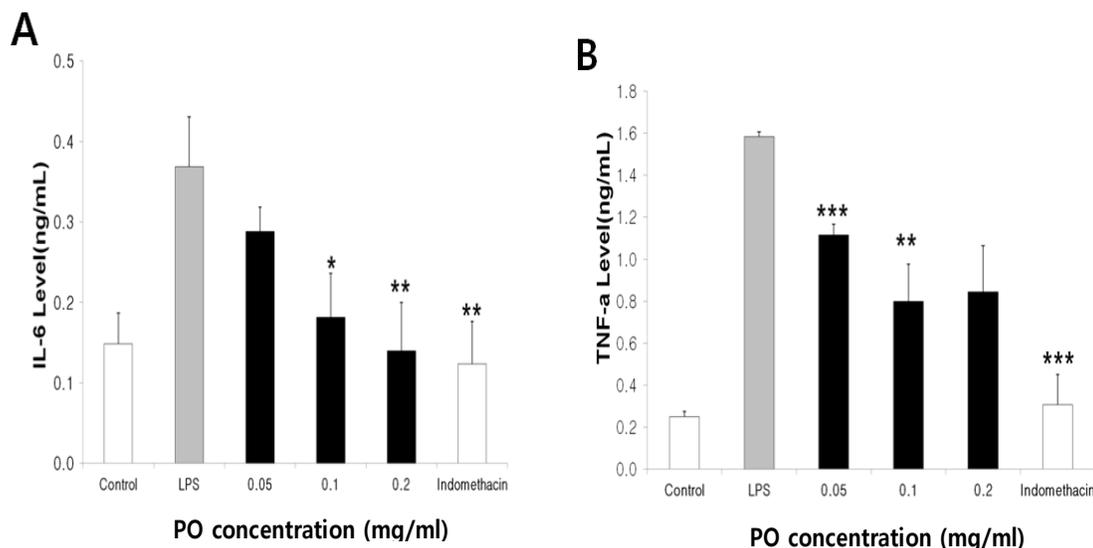


Figure 2. Effects of PO on (A) IL-6 and (B) TNF- α production. RAW 246.7 cells were treated with LPS (1 μ g/ml) with and without PO (0.05-0.2 mg/ml), or indomethacin (0.25 mM) for 3 hrs prior to TNF- α and IL-6 concentration being measured. Values are the mean \pm SEM of the three independent experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with LPS-stimulated group.

not well known. They are involved in bone resorption, IL-6 and TNF- α elevated levels can be found in many acute and chronic inflammatory diseases (Diehl et al., 2002). Figure 2A and B showed that LPS significantly stimulated IL-6 and TNF- α production. PO and indomethacin have only slight influence on TNF- α level inhibition in LPS stimulated RAW 264.7 cells. The results showed that the inhibition of IL-6 synthesis were 0.29 ± 0.03 , 0.18 ± 0.05 and 0.14 ± 0.06 ng/ml, for 0.05, 0.1 and 0.2 mg/ml, respectively. The inhibition LPS-induced increases in IL-6 and TNF- α has been used to impose the potential anti-inflammatory effects of drug (Kumar et al., 2004) and medicinal herb (Kim et al., 2005). In addition, IL-6 plays a pivotal role in controlling the immune system so IL-6 has become a promising outstanding target for immunomodulatory anti-rheumatic therapy (Nishimoto and Kishimoto, 2006).

Although the precious mechanisms regulating the anti-inflammatory activity of PO are not yet known, in this study it was demonstrated that PO inhibits LPS-induced production of pro-inflammatory mediators including NO, PGE₂, iNOS, IL-6 and TNF- α in RAW 264.7 mouse macrophages for the first time. These results suggest that PO possesses anti-inflammatory properties and could control macrophage-mediated inflammatory stimulation. Although the detailed mechanism remains to be elucidated, the PO may be a potential candidate as a remedy against inflammatory disorders such as rheumatoid arthritis.

Conclusion

Although the important mechanisms regulating the anti-inflammatory activity of PO is not yet known, in this research it was shown that PO inhibits LPS-stimulated production of pro-inflammatory mediators in RAW 264.7 mouse macrophages. These findings suggest that PO has anti-inflammatory effects and could regulate macrophage-mediated inflammatory stimulation. Further studies are needed to verify the precious mechanism regulating anti-inflammatory activities of PO.

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Conflicts of interest

Authors declare that there are none.

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Full Length Research Paper

Content and chemical composition of the essential oil from *Byrsonima verbascifolia* Rich. ex a. Juss. collected in different seasons and times of day

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Certain factors may influence the special metabolite production in plants. Leaves of *Byrsonima verbascifolia* (Malpighiaceae) were collected during different seasons and times of day to determine the concentration and chemical composition of volatile oils. Chemical analysis indicated that oxygenated sesquiterpenes were the most concentrated, and oxygenated monoterpenes were the least concentrated. The primary components of the essential oil were pentacosane (2.747 to 9.613%), spathulenol (3.398 to 10.552%), and benzene-1,2-dicarboxylic acid diethyl ester (3.861 to 15.307%). Seasonal and circadian variations did not influence the essential oil content. However, the essential oil's chemical composition was influenced by seasonal variation, among them the spathulenol.

Key words: Murici, seasonality, circadian variation, terpens, leafs.

INTRODUCTION

The use of phytotherapeutic agents has increased remarkably in Brazil and worldwide not only from incentives by the World Health Organization (WHO), but also to search for therapeutic alternatives with fewer side effects and lower cost (WHO, 2007; Yunes and Calixto, 2001). Cerrado is the second largest Brazilian biome in diversity and comprises over 7,000 species (Almeida et al., 1998). Given such diversity, several compounds may have active components with therapeutic activity, such as secondary or special metabolites particularly essential oils. However, environmental and physiological factors might interfere not only with the content, but also with the

quality of such substances; thus, processing by the cosmetic, food, and phytotherapeutic industries may be difficult (Zaroni et al., 2004; Kutchan, 2001). Circadian rhythm and seasonality are among the factors that may interfere with essential oil production because the nature and yield of their constituents may not be consistent throughout the year (Gobbo-Neto and Lopes, 2007).

The genus *Byrsonima* comprises approximately 150 species; 60 are found in Brazil (Judd et al., 1999; Castro and Lorenzini, 2005) distributed across the federal district and the following states: Mato Grosso, Mato Grosso do Sul, São Paulo, Minas Gerais, Goiás, Bahia, Tocantins,

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and Paraíba (Vieira et al., 2010). Such plants are known as “murici”, “murici-pequeno”, “murici-rasteiro”, and “orelha-de-veado”, and they are used to prepare fruit juices, liqueurs, popsicles, and jellies; they are also used in traditional medicine (Camargos et al., 2001). These plants are traditionally used against asthma, fever, and skin infections, and the bark produces antidiarrheal and astringent effects (Caceres et al., 1993; Brandão, 1991). The branch leaves have antisyphilitic, diuretic, and emetic properties. Food and pharmaceutical industries employ the oil extracted from seeds (Faria et al., 2002). Among the 24 plant extracts traditionally used in Colombia to treat skin affections, methanolic extract from *Byrsonima verbascifolia* Rich. Ex A. Juss. produces the most potent antimicrobial and antiviral activity (Lopez et al., 2001).

Phytochemical studies conducted on *B. verbascifolia* identified phenolic and terpenic compounds, including tannins, flavonoids, and triterpenes in the leaves and bark (Lorenzi et al., 2002). Although certain flavonoid derivatives were isolated from *Byrsonima* plants, triterpenes are the most frequently occurring class of natural substances in this genus. Seven triterpenoid constituents were isolated from *B. verbascifolia* stem bark through hexane extraction (Gottlieb et al., 1975). Sulfonoglycolipids, steroids, triterpenes, aromatic esters, amino acids, and proanthocyanidins were reported in *Byrsonima crassifolia*, *Byrsonima microphylla*, and *B. verbascifolia* (Sannomiya et al., 2005).

Ethnopharmacological use of these plants has generated increased interest in identifying the chemical constitution and pharmacological potential for species in the genus *Byrsonima*. However, from approximately 150 of such species, only 13 have been extensively studied; most studies were not continued, and the metabolites associated with traditionally attributed activities have not been identified (Guilhon-Simplício et al., 2005). Such studies identified primarily phenolic compounds using high-performance liquid chromatography, whereas studies that focused identifying terpenoids using gas chromatography-mass spectrometry (GC-MS) were few, especially for extracts from leaves in the species *B. verbascifolia*. Therefore, the aim of this study was to establish the chemical composition for the essential oil in leaves from *B. verbascifolia* Rich. ex A. Juss. and assess the circadian as well as seasonal variability of its content and chemical composition.

MATERIALS AND METHODS

Plant

Adult *B. verbascifolia* leaves were collected from a native population in Rio Verde County-GO (18°02'02.6" S and 50°57'10.1" W and 771 m altitude). Voucher specimen (code number HJ 5643) was deposited at the Herbarium Jataiense of the Universidade Federal de Goiás, Jataí, GO. Six individual plants were established and comprised two specimens from each block, which were 50 m apart. The specimens were collected in the first week of each month

(December, 2010 to November, 2011) from three blocks at three different times of day (6:00, 12:00, and 18:00 h) and clustered according to the seasons. The seasons were distributed as follows: spring (October to December), summer (January to March), fall (April to June), and winter (July to September). The soil moisture was assessed through a gravimetric method when each of the 12 monthly samples were collected using one soil sample per block, which was acquired from the zero-to-20 cm depth layer on the same day the leaf samples were collected at 12:00 h.

Leaves were transferred to the Natural Products Section in the Laboratory of Plant Tissue Culture at Instituto Federal Goiano, Câmpus Rio Verde, GO, air-dried at 35°C and ground to fine powder. The source meteorological was obtained in the meteorological station INMET/Universidade de Rio Verde.

Extraction of essential oils

Dried (50 g) material was subjected to hydrodistillation (800 ml distilled water) for 2 h using a Clevenger-type apparatus. The crude oils were extracted with CH₂Cl₂, dried (anhydrous Na₂SO₄), filtered, and the solvent removed at retention time. Oil samples were kept in amber glass vials at 4°C until the identification of their chemical composition. The extraction yield of each essential oil was expressed in % (w/w) of the dried leaves.

Chemical analysis of the essential oils

Chemical analysis was performed in the Chemistry Department at the Universidade Federal de Lavras, Lavras, MG. The essential oils were analyzed with a Shimadzu QP5050A apparatus equipped with a mass selective detector operating by electronic impact (70 eV) and a DB-5 cap. column (30 m × 0.25 mm i.d., film thickness 0.25 mm). Helium (1 ml.min⁻¹) was used as carrier gas. The oven temperature was programmed rising from 60 to 240°C at 5°C.min⁻¹, a 10°C.min⁻¹ from 240 to 270°C and then held isothermal at 270°C for 5 min; injector temperature, 220°C; detector temperature, 240°C; the sample injection volume was 1.0 µl and diluted in dichloromethane at a 1:20 injection ratio. The tests were performed in triplicate in scan mode at 2.0 scans/s and 45 to 500 m/z.

Volatile compounds were identified by comparing the resulting mass spectra with records from the Wiley and FFNSC 1.2 computational libraries. These compounds were also determined using the retention indices (RI) (Van den Dool and Kratz, 1963), from a series of n-alkanes of (C₈-C₄₀) under the same chromatographic conditions that were used for the essential oils. The resulting values were subsequently compared with the Kovats indices that are available in the literature (Adams, 2007).

Statistical analysis

To establish the content and chemical composition of the essential oil in the leaves and new branches on *B. verbascifolia*, the study design included randomized blocks with a 4 × 3 factorial design as well as three replications, and it corresponded to the 12 monthly samples as well as the three different times of day for sample collection. The data were subjected to analysis of variance, and the means were compared at 5% probability using software SISVAR-System for Analysis of Variance (Ferreira, 2007).

RESULTS AND DISCUSSION

Essential oil content

The essential oil in dry leaves from *B. verbascifolia* was a

Table 1. Essential oil content (EOC) in leaves from Murici (*Byrsonima verbascifolia* Rich. ex A. Juss.) collected in different seasons and times of day, Instituto Federal Goiano, Câmpus Rio Verde, GO, 2012.

Seasons	Spring	Summer	Fall	Winter
EOC (%)	0.003 ^a	0.005 ^a	0.004 ^a	0.005 ^a
Time of day (h)				
	6:00	12:00	18:00	
EOC (%)	0.005 ^a	0.003 ^a	0.004 ^a	

^aMeans followed by the same letter are not significantly different using a Scott-Knott test at 5% probability.

slightly yellowish fluid with low viscosity and a poorly characteristic odor. The season and time of day did not alter the essential oil content. The season and time of day did not significantly influence essential oil content in separate analysis; the oil content varied from 0.003 to 0.005% in both instances (Table 1). The oil content did not change in a study that assessed the influence of irradiation levels on *Hyptis marrubiodes* Epl. essential oil yield (Sales et al., 2009).

Based on the results produced under the conditions in Rio Verde County, GO, one might suggest that samples may be collected in any season at the three investigated times of day. Although the phytomass yield was not analyzed, the plants produced more leaves in spring and summer, which might increase essential oil yield during these seasons.

Chemical analysis

The chemical composition of the essential oils from leaves of *B. verbascifolia* varied among the seasons investigated (Table 2). Certain substances (isobutyric acid and β -bisabolene, among others) were not detected in every season, likely from seasonal variations and the influence of abiotic factors, such as light. A study assessing the influence of irradiation levels on essential oil chemical composition in *H. marrubiodes* Epl. found that the oil components iso-3-thujanol and δ -cadinene varied (Sales et al., 2009).

The compounds nerolidol, benzene-1,2,-dicarboxylic acid diethyl ester, spathulenol, (Z,Z)-farnesol, caryophyllene oxide and pentacosane were identified in concentrations over 1% in each season investigated. Spathulenol was identified in the epicuticular layer of the leaves in several species, such as *Byrsonima linearis*, and it demonstrated insecticide activity as well as protection against desiccation from dissipation of excess light (Faini et al., 1999; Silva et al., 2006). Nerolidol is mentioned because it is an absorption enhancer that acts by reinforcing the skin bilayers through orientation along the stratum corneum lipids (Marinho, 2008; Williams and Barry, 2004).

Forty-eight chemical components were identified in the essential oil from *B. verbascifolia* in different seasons

with relative concentrations of oxygenated monoterpenes that varied from 2.782 to 17.386%; oxygenated sesquiterpenes from 22.496 to 45.646%, sesquiterpene hydrocarbons from 3.437 to 31.430%; and additional components, including alcohols, acids, hydrocarbons, long-chain hydrocarbons, and aldehydes, that varied from 18.613 to 49.088%.

Sesquiterpenes predominated except for winter when components such as alcohols and hydrocarbons prevailed. For sesquiterpene hydrocarbons, the highest content was observed in spring (27.82%) and summer (31.43%). With the earliest rainfalls at the beginning of spring and consequent increase in soil moisture, new shoots (Figure 1A) and the first flowers appeared. Full blossoming began after monthly precipitation became greater than 250 mm and lasted until the end of the season (Figure 1B). The times of day for sample collection (6:00, 12:00, and 18:00 h) did not produce a difference in sesquiterpene hydrocarbons (20.84, 16.75 and 14.32%, respectively). For oxygenated sesquiterpenes, the greatest percentage was observed in the fall (45.64%) when the temperature had dropped, whereas the oil content was not dependent on the time of day of sample collection (29.31, 32.2 and 29.01%).

The highest percentage of oxygenated monoterpenes was observed in spring and summer (15.20 and 17.38%, respectively) as described in Table 3. More leafing, fruit formation, and fully formed fruits were observed in the spring when the average temperature was over 23°C, the air relative humidity above 70%, the average monthly precipitation over 20 mm, and the soil moisture over 10% (Figure 2A, B, C, and D). The soil moisture remained high from spring to summer and fell below 5% in winter when rainfall is virtually absent. Fruit maturation (Figure 1C) was observed at the end of spring and during summer. The relative concentrations (30.93, 29.11 and 32.72%) of oxygenated monoterpenes did not differ with the time of day for sample collection (6:00, 12:00, and 18:00 h).

The combined content for the remainder of classes (alcohols, esters, hydrocarbons, and others) was greater in the dry season (winter). During this season, the relative air humidity was below 50%, the soil moisture below 5% (Figure 2B and D), and the leaves were yellowish or fully dry, which facilitated senescence. Certain leaves contained chestnut brown-reddish lesions, which may

Table 2. Chemical composition of the essential oil in leaves from Murici (*Byrsonima verbascifolia* Rich. ex A. Juss.) collected from December/2010 to November/2011. The compounds are listed in accordance with the elution order from a DB-5 column. Instituto Federal Goiano, Câmpus Rio Verde, GO, 2012.

Compound	Classification	RI*	Spring	Summer	Fall	Winter	\bar{X}
(Z)-3-hexen-1-ol	Alcohol	849	2.243	5.880	0.755	-	2.220 ^c
Butanoic acid butyl ester	Ester	948	3.327	0.785	-	0.004	1.029 ^c
Octen-3-ol	Alcohol	981	-	0.143	-	-	0.036 ^c
Butanoic acid (butyl) ester	Ester	990	3.307	0.109	1.570	0.402	1.347 ^c
(Z)-linalool oxide	OM	1064	1.040	7.431	0.220	0.008	2.175 ^c
Linalool	OM	1098	4.681	2.132	0.591	2.038	2.361 ^c
3,7-dimethyl octanol	Alcohol	1189	0.196	2.339	5.961	5.758	3.564 ^b
α -Terpineol	OM	1190	0.639	0.652	0.192	0.316	0.450 ^c
Decanal	Aldehyde	1200	-	0.051	-	-	0.013 ^c
Isobutyric acid	Acid	1211	-	-	-	1.046	0.262 ^c
Nerol	OM	1225	-	0.492	0.455	0.134	0.270 ^c
Geraniol	OM	1270	8.301	5.948	0.959	0.904	4.028 ^b
Eugenol	PP	1359	0.546	0.731	0.366	0.834	0.619 ^c
Decanoic acid	Acid	1363	-	0.069	0.090	0.007	0.041 ^c
Undecanol	Alcohol	1364	0.044	-	-	0.463	0.127 ^c
Cyclosativene	SH	1368	0.138	0.287	0.202	0.006	0.158 ^c
β -Bourbonene	SH	1381	0.979	0.137	0.021	0.036	0.293 ^c
β -Elemene	SH	1390	2.881	1.566	0.036	0.180	1.166 ^c
α -Himachalene	SH	1442	9.389	8.874	0.850	1.511	5.156 ^b
α -Humulene	SH	1452	1.234	1.266	0.040	0.008	0.637 ^c
(E)- β -farnesene	SH	1459	1.258	2.005	0.138	0.331	0.933 ^c
Germacrene D	SH	1464	0.853	0.216	0.054	0.073	0.299 ^c
Aromadendrene	SH	1471	1.133	1.805	0.796	1.633	1.341 ^c
Viridiflorene	SH	1478	0.366	0.167	0.210	0.067	0.202 ^c
β -selinene	SH	1478	0.666	0.437	0.149	0.032	0.321 ^c
Bicyclogermacrene	SH	1489	8.294	10.149	0.847	1.093	5.096 ^b
β -Bisabolene	SH	1500	-	2.937	-	0.053	0.748 ^c
α -Bulnesene	SH	1509	-	0.434	0.041	1.514	0.497 ^c
δ -Cadinene	SH	1514	-	0.566	0.018	0.002	0.147 ^c
Nerolidol	OS	1530	7.306	6.898	5.000	3.509	5.678 ^b
α -Elemol	OS	1542	0.101	0.361	4.010	6.129	2.650 ^c
Germacrene B	SH	1547	0.638	0.584	0.036	0.009	0.317 ^c
Benzene-1,2-dicarboxylic acid diethyl ester	Ester	1557	3.401	5.797	10.552	7.925	6.919 ^a
Spathulenol	OS	1573	3.863	5.936	15.309	4.755	7.466 ^a
Caryophyllene oxide	OS	1573	3.722	5.650	3.201	2.666	3.810 ^b
Viridiflorol	OS	1579	0.724	-	1.201	1.069	0.748 ^c
α -Bisabolol	OS	1664	0.068	0.108	0.019	0.036	0.058 ^c
Heptadecane	HC	1691	-	0.061	0.161	0.175	0.099 ^c
Heptadecanol	Alcohol	1698	1.983	0.138	3.057	8.459	3.409 ^b
(Z,Z)-farnesol	OS	1711	8.620	2.052	4.333	4.835	4.960 ^b
(Z) lanceol	OS	1752	0.118	0.683	0.150	0.129	0.270 ^c
10-Epi- γ -eudesmol	OS	1770	-	0.809	12.423	4.923	4.539 ^b
Octadecane	HC	1789	-	0.219	-	0.338	0.139 ^c
Hexadecanol	Alcohol	1860	0.105	0.232	0.039	0.973	0.337 ^c
Hexadecanoic acid (palmitic acid)	ACID	1964	0.692	-	-	2.078	0.692 ^c
Eicosane	LCHC	1995	0.222	0.043	0.099	1.169	0.383 ^c
Tetracosane	LCHC	2389	0.140	0.000	-	1.336	0.369 ^c
Pentacosane	LCHC	2489	8.410	2.748	9.613	18.954	9.931 ^a
Identified total			91.628	89.929	83.763	87.918	88.310

Table 2. cont'd.

Oxygenated monoterpenes (OM)	15.207 ^a	17.386 ^a	2.782 ^b	4.234 ^b	9.902
Sesquiterpenehydrocarbons (SH)	27.829 ^a	31.431 ^a	3.437 ^b	6.546 ^b	17.311
Oxygenated sesquiterpenes (OS)	24.522 ^b	22.498 ^b	45.646 ^a	28.050 ^b	30.179
Other (aldehydes, alcohols, etc.)	24.071 ^b	18.614 ^b	31.898 ^b	49.088 ^a	30.918

RI*: Experimental retention index using DB-5 column. -: non-detected component. Number of injections per season: 9. Standard deviation: ± 0.33 . HC: hydrocarbon LCHC: long-chain hydrocarbon; PP: phenylpropanoid; OM: oxygenated monoterpene; SH: sesquiterpene hydrocarbon; SO: oxygenated sesquiterpene.



Figure 1. *Byrsonima verbascifolia* Rich. ex A. Juss. A: On set of sprouting in the spring. B: On set of flowering in the spring. C: Fruits ripening in the summer. Photos: Henrique Antonio de Oliveira Lourenço.

have been related to a biological/physiological response. These lesions are a common trait in this species and may be observed during winter for all of the plants in the area investigated.

The three main components of the essential oil in *B. verbascifolia* Rich. ex A. Juss leaves include a long-chain hydrocarbon (pentacosane), an ester (benzene-1,2-dicarboxylic acid diethyl ester), and an oxygenated

sesquiterpene (spathulenol). Although season and time of day did not contribute to such compounds, greater spathulenol content was observed in fall (15.30%), when the soil moisture had not decreased but rainfall, average temperature, and air relative humidity were lower (Figure 2).

Certain studies have reported a variation in essential oil chemical composition as a function of seasonal variation.

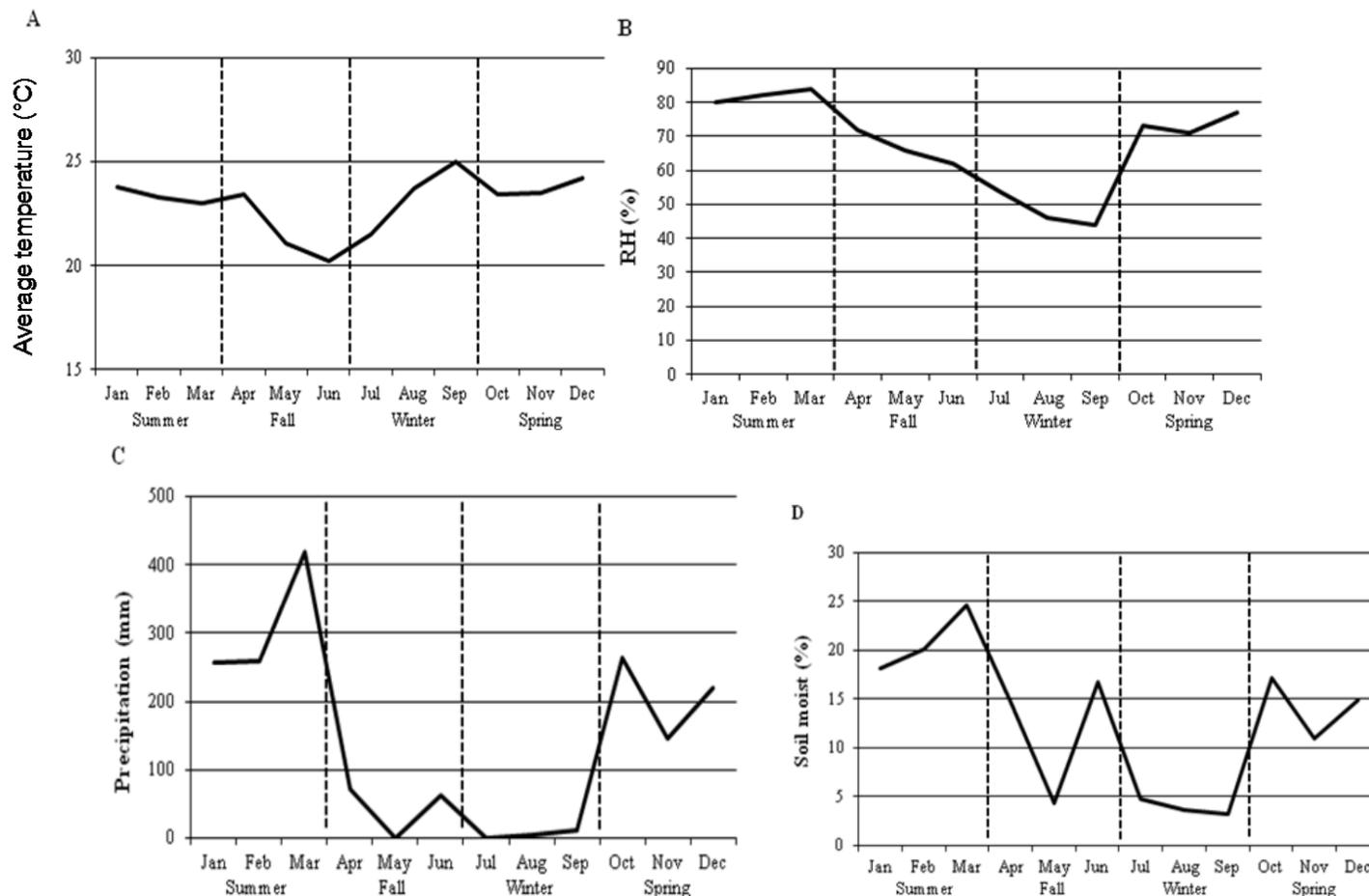


Figure 2. Meteorological data and soil moisture (monthly averages) from December 2010 to November 2011 in Rio Verde County (GO). A: Average temperature (°C). B: Air relative humidity (%). C: Precipitation (mm). D: Soil moisture (%).

In the species *H. marruboides* Epl. (Lamiaceae), although non-oxygenated (cadalene, germacrene D, α -copaene, and α -caryophyllene) and oxygenated (caryophyllenol and cedrol) sesquiterpenes are observed at much lower levels in the essential oil, they were quantitatively different depending on the season (Botrel et al., 2010). The primary compounds concentrations in the essential oil from *Elyunurus muticus* (Sprengel) O. Kuntze (Poaceae), e.g., (E)-caryophyllene, spathulenol, bicyclogermacrene, and caryophyllene oxide, varied as a function of season the plants were collected (Hess et al., 2007).

The content of the pentacosane (9.53, 11.26 and 8.99%), benzene-1,2-dicarboxylic acid diethyl ester (6.41, 6.71 and 7.64%) and spathulenol (8.93, 6.36 and 7.11%) did not vary with the time of day (6:00, 12:00 and 18:00 h) that samples were collected. Studies using samples from the wild species *Eremanthus seidelii* (Asteraceae) collected in its natural habitat found that the concentration of secondary metabolites was constant, which suggests that environmental factors do not

systematically influence the production of such compounds (Sakamoto et al., 2005; Gobbo-Neto and Lopes, 2007).

Conclusions

Essential oil from *B. verbascifolia* Rich. ex A. Juss leaves is composed of oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes; the largest fraction is oxygenated sesquiterpenes and the smallest is oxygenated monoterpenes. The primary components of the essential oil were pentacosane (9.93%), spathulenol (7.46%), and benzene-1,2-dicarboxylic acid diethyl ester (6.92%).

Neither seasonal nor circadian variation influenced the essential oil content. The terpene class was influenced by seasonal variation; the percentage of oxygenated monoterpenes and sesquiterpene hydrocarbons was the highest in spring and summer, and the highest percentage of oxygenated sesquiterpenes observed in

fall. Circadian variations were not observed for this class. Among the primary compounds, only spathulenol was influenced by seasonal variation; its highest relative concentration was observed in fall.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Note on somatic embryogenesis and synthetic seed production in *Angelica glauca*: A valuable medicinal plant of Himalaya

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This is the first report of somatic embryogenesis and synthetic seed production in *Angelica glauca*, a valuable medicinal plant of Himalaya. Somatic embryogenesis protocol was developed using leaf explants from newly sprouted rhizomes. Mature leaf explants were inoculated in Murishige and Skoog (MS) medium supplemented with 3 μM 2,4-dichlorophenoxyacetic acid (2,4-D) induced 86% callus. Calli (0.5 g) subcultured into different concentration of 1-naphthaleneacetic (NAA) and 6- benzylaminopurine (BA) either alone or in combination produced globular structure and their differentiation. NAA (2 μM) in combination of BA (2 μM) germinated 2.2 shoots per culture in 8 weeks time. Plantlets when transferred into sand, soil and peat moss (1:1:1) ratio were found suitable for acclimatization and 75% plantlets survived. Somatic embryos were put into a combination of 3% (w/v) sodium alginate and 100 μM calcium nitrate for 30 min for protecting the somatic embryos and the production of synthetic seeds. Experiments on the evaluation of synthetic seeds are under progress.

Key words: Himalaya, *Angelica glauca*, somatic embryogenesis, synthetic seed.

INTRODUCTION

Angelica glauca Edgew. (Family Apiaceae), is a perennial, glaucous, aromatic and medicinal herb, distributed across the Indian Himalaya (from north-west to east Himalaya) between 1800 and 3700 m asl (Anonymous, 1985). Its aromatic root is used as a spice by indigenous communities (Collet, 1980) and considered cardioactive, cordial and useful in constipation, rheumatism and urinary disorders (Anonymous, 1985). The powdered root with milk is given in bronchitis (Gaur, 1999) and also useful in stomach ailments and female

remedies and therefore known as female ginseng.

The root of the species consist of many important secondary metabolites of pharmaceutical importance (Wang et al., 1996) and is preferred the utilizable part. The indigenous communities of the region harvest the root of the species not only for their domestic use but also for trade, which is effectively ending the possibility of future growth and regeneration of the species. Also, overgrazing in the alpine/sublpine forests has contributed for alteration in the habitat of the species (Sundriyal, 1995;

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Joshi et al., 2001). All these factors placed the species in Indian Red Data Book (Jain and Sastry, 1984) and ranked number three in the list of medicinal plants prioritized for consultation (Sastry and Chatterjee, 2000). The herbaria records also indicate that the species has reached critically endangered status in the Himalaya (Anonymous, 1997).

Tissue culture has emerged as an important method for conservation of threatened species and enhancement of secondary metabolites (Balachandran et al., 1990; Wawrosch et al., 2001). Reports suggest that clonal material possesses similar genetic constitution similar to mother plant (Hartmann and Kester, 1983). Therefore, clonal propagation through tissue culture techniques can be a best way to enhance the growth rate, reduction in time for the preparation of planting material and production of large number of propagules starting from a single explant (Bonga, 1982). Besides, reports on secondary metabolite production by somatic embryo culture in *Ammi vishaga* (Apiaceae) provide an encouraging lead for trying the same in *A. glauca*. This could effectively reduce the pressure of raw material from pharmaceutical houses (Hiraoka et al., 2004). In this regard, it is further encouraging that the amount of secondary metabolites produced by somatic embryos is proportional or sometimes higher than the potential plant parts in some cases (Kaul and Staba, 1967). Also, encapsulation of somatic embryos for the production of synthetic seeds has emerged as advancement and received attention of several workers (Bapat and Rao, 1988).

Keeping this in view, the present study first time describe the somatic embryogenesis protocol for mass multiplication, synthetic seed production and base material for the secondary metabolite production in *A. glauca*.

MATERIALS AND METHODS

Plant and sterilization

Rhizomes of *A. glauca* were collected from Fojal (Parvati valley-2660 m asl, Himachal Pradesh, India) in August 2000. The rhizomes were maintained in earthen pots in the nursery of G.B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora (1240 m asl). Mature leaf explants (3 × 2 cm) were excised from fresh sprouts (20 to 25 days old) at 4 to 5 leaf stage for initiating tissue culture work. Leaves were thoroughly washed with tap water for 15 min followed by Tween 20 (2% v/v) for 15 min and again washed with distilled water. Sterilization was conducted using aqueous solution of 0.1% (w/v) mercuric chloride for different time durations (0, 5, 10, 20 and 30 min). Treated material was repeatedly washed and agitated (at least 5 times) with sterile distilled water under aseptic Laminar Flow System to remove any traces of HgCl₂.

Media preparation and culture conditions

All the explants were inoculated into Murishige and Skoog (MS)

medium containing 3% (w/v) sucrose and 0.7% (w/v) agar (Qualigens, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 22 min. All the chemicals used were of analytical grade (Sigma chemicals Co., St. Louis, USA; Merck, Hi-Media and Qualigens, Mumbai, India). The culture tubes (25 × 150 mm; Borosil India Ltd., Mumbai, India) containing 15 ml MS medium were plugged with non absorbent cotton wool plugs. Cultures were maintained at 25±1°C in 16 h light and 8 h dark cycle with irradiance (40 μ moles m⁻² s⁻¹) by cool fluorescent tubes (Philips, India).

Embryogenic callus induction

Surface sterilized leaf explants (2 × 2 mm), were placed in the medium with abaxial sides touching the medium for callus induction. Callusing medium consist of MS basal salts (Murashige and Skoog, 1962) containing 3% sucrose and 0.7% agar supplemented with different combination of 1-naphthaleneacetic (NAA) (0, 1, 2, and 4 μM) and 6- benzylaminopurine (BA) (0, 1, and 2 μM). After 4 weeks, once the explant had induced callus, the frequency and quality of callus formation was recorded. One set of explant cultured on MS medium without growth regulator served as control.

After the mass production in the medium containing 3.0 μM, 2,4-dichlorophenoxyacetic acid (2,4-D; best responding treatment) calli were then transferred to different concentration of NAA (0, 0.5, 1, 2, and 4 μM) and BA (0, 1, and 2 μM) for somatic embryogenesis. Bipolar structures were scored as somatic embryos. Data on frequency of somatic embryogenesis were recorded after 8 weeks. Somatic embryos, thus formed, were further transferred to combinations of different concentrations of NAA (0.5, 1.0, 2.0 and 4.0 μM) with 2 μM BA and the control treatment (0 μM NAA+0 μM BA) for germination and subsequent plantlet formation. Regenerates with well developed leaves were recorded as shoots. Data on shoot and root formation were recorded after 8 weeks.

Thus the whole experiment up to the formation of plantlets through various stages of callus formation (4 weeks), somatic embryo formation (8 weeks) and plantlet formation (8 weeks) took a period of 20 weeks, that is, 5 months in all.

Encapsulation

For synthetic seed production, 4 to 6 mm long somatic embryos were removed from embryo cultures and dipped in 3% (w/v) autoclaved sodium alginate solution. The embryos were agitated 5 min and then picked up using Pasteur pipette and placed into sterile 100 mm calcium nitrate for 30 min (standardized). During this time occasional shaking was done. The resulting beads were recovered by decanting the calcium nitrate and then washed thoroughly with autoclaved distilled water (Janeiro et al., 1997). Embryos were cultured in MS hormone free medium and incubated in growth chamber (condition described earlier). For each treatment, five embryos with three replicates were used. Data on embryo germination were scored after 4 weeks of incubation.

Acclimatization

For acclimatization, complete plantlets (60 No.) were removed from culture tubes and washed thoroughly in running tap water to remove the adhering medium prior to transformation in plastic pots (5 cm diameter) containing substrate (120 g w/v), a mixture of autoclaved soil, sand and peat moss (1:1:1 ratio). Potted plantlets were kept initially in a growth chamber. Transparent polybags were inverted over each plantlet (for up to 30 days) to maintain high humidity and irrigated by ¼ MS basal salt solution (< 1 week)

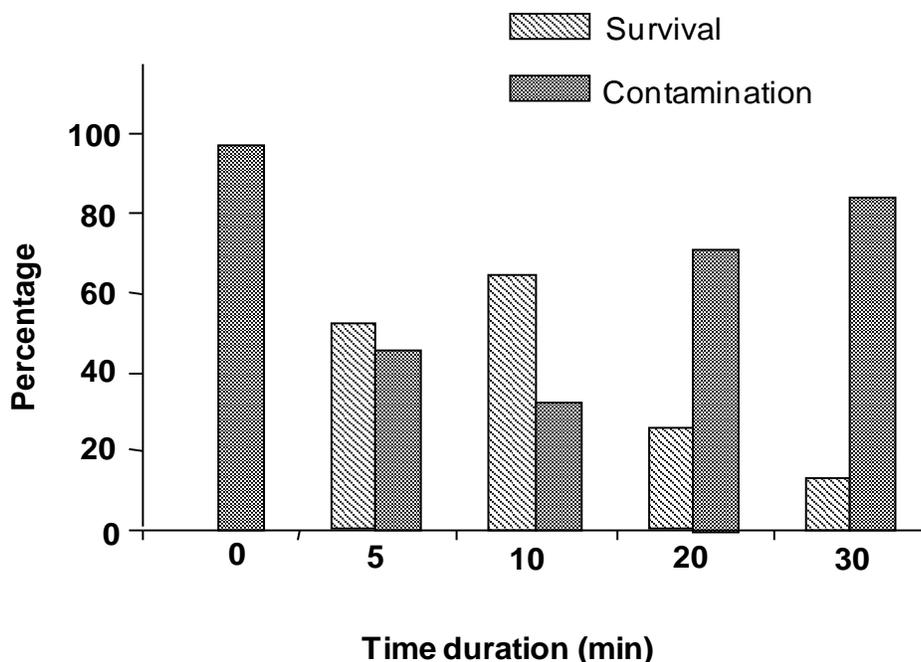


Figure 1. Effect of HgCl_2 on percent survival of *Angelica glauca* explants.

followed by tap water on alternate days. After 2 weeks monitoring in growth chamber when new leaves appeared plantlets were transferred to polyhouse. The covers were permanently removed and hardened plantlets (45 No.) were transferred to nursery condition.

Statistical analysis

All experiments were set up in completely randomized block designs (8x3 test tubes per treatment). Data obtained were analyzed statistically using computer package SYSTAT (Wilkinson, 1986). Significant difference ($P < 0.05$) among means were tested by Fischer's Least Significant Difference (Snedechor and Cochran, 1968). All experiments were repeated twice.

RESULTS

An efficient protocol for somatic embryogenesis and synthetic seed production has been developed for *A. glauca*. The highest percentage of aseptic cultures (66.3%) was obtained using HgCl_2 for 10 min (Figure 1). Further increase in time duration in HgCl_2 drastically reduced the percentage survival of the explants.

Of the various concentration of 2,4-D tested, 3 μM 2,4-D resulted in significantly ($P < 0.05$) higher frequency (86.7%) of callus formation (Table 1 and Figure 2A). The initiation of callus was also quick in 3 μM 2, 4-D (data not shown) as compared to others. Light cream callus was obtained from the cut end of leaves after 4 weeks of culture.

Further increase in 2, 4-D concentration considerably

reduced the frequency of callus induction. The control set could not produce the callus, however, browning and wilting was observed.

When 4 weeks old calli were transferred on MS medium supplemented with different combinations of NAA and BA, somatic embryos emerged from the peripheral areas of the callus mass (Figure 2 B and C). NAA and BA (2 μM each) responded significantly ($P < 0.05$) better to other concentrations and 100% cultures showed embryonic callus (Table 2). When compared to control, callus did not turn to embryonic callus. As the differentiation progressed, the embryo appeared from other parts as well. Most of the embryos expanded sequentially and protruded to form shoots to roots. The two leaf sheaths enlarged and elongated (Figure 2D). At maturity, the basal region of the embryo contracted and whole structure was easily dislodged from each other and the parent cell. Among different combinations, BA and NAA (2 μM each) proved to be the best and produced 2.2 shoots per culture (Table 3).

Somatic embryos encapsulated with 3% sodium alginate along with 2% calcium chloride formed uniform size beads (Table 3 and Figure 2E). Capsules formed with 1 and 2% sodium alginate were too soft and fragile to handle. Whereas, those formed with 4% sodium alginate were too hard and stiff that they hindered the emergence of root and shoot primordia.

Observations for acclimatization experiments revealed that well rooted plantlets when transferred to autoclaved sand, soil and peat moss mixture (1:1:1 ratio), 75% plantlets survived in nursery condition (Figure 2F). A comparison

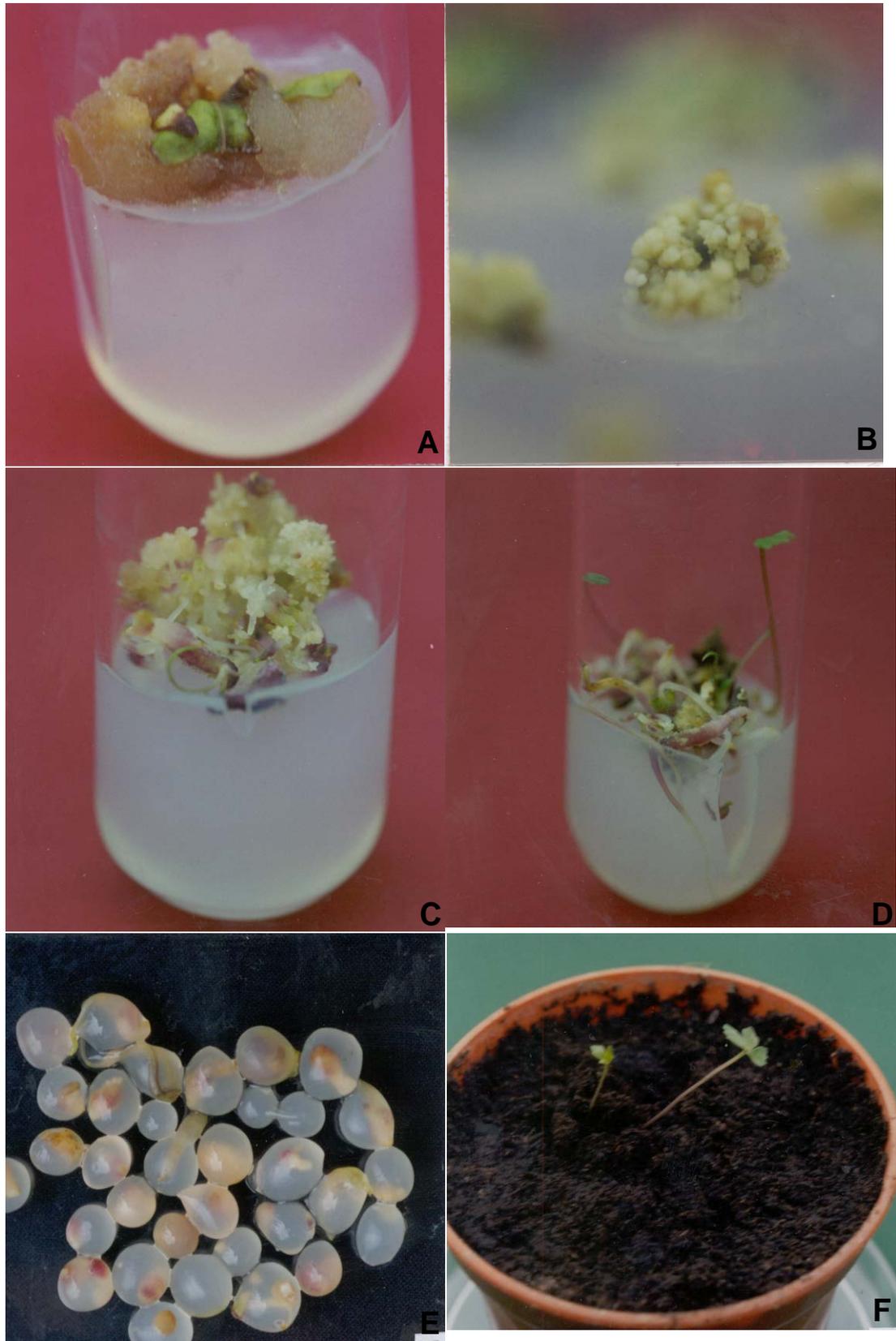


Figure 2. Different stages of somatic embryogenesis in *Angelica glauca*; A: Callus induction, B-C: embryogenesis, D: Plantlet formation, E: synthetic seeds, and F: acclimatization of *in vitro* plants.

Table 1. Effect of 2,4-D on callus formation after 4 weeks of incubation.

2,4 D (μM)	Callus (%)	Callus colour and texture
0.0	0.0	-
1.5	33.3	Light cream, friable
3.0	86.7	Light cream, friable
6.0	20.0	Light cream, friable
12.0	6.67	Light cream, friable
F (P<0.05)	33.5	-

Table 2. Effect of hormonal combination on embryogenesis of *Angelica glauca* (Data recorded after 8 weeks of culture).

Hormonal combination (μM)		Embryonic callus (%)
NAA	BAP	
0.0	0.0	0.0
0.5	1.0	33.3
1.0	1.0	53.3
2.0	1.0	40.0
4.0	1.0	26.6
0.5	2.0	60.0
1.0	2.0	73.3
2.0	2.0	100.0
4.0	2.0	66.6
LSD (P<0.05)	-	2.8
F (P<0.05)	-	87.8

Table 3. Effect of PGR combinations on maturation of somatic embryos of *A. glauca*.

Hormonal combination		Number of shoots/culture	Induction of leaf (%)	Root induction (%)
NAA	BAP	-	-	-
0.0	0.0	0.0	0	0
0.5	2.0	1.0	13.33	46.66
1.0	2.0	1.5	26.66	60.0
2.0	2.0	2.2	20.0	6.67
4.0	2.0	0.6	13.33	60.0
LSD (P<0.05)	-	2.75	-	-
F (P<0.05)	-	34.26	-	-

of available tissue culture protocols for

Angelica species (Table 4) reveals that the present protocol for *A. glauca* has an advantage in terms of multiplication potential and long term utilization of synthetic propagules.

DISCUSSION

Sterilization of the explants plays a crucial role with respect to the success of the experiment. Mercuric

chloride (HgCl_2) is used in controlling several microbial infestations (Bonga and Aderkas, 1992). Treatment of HgCl_2 in the present study for >10 min proved to be deleterious. This could be attributed to its penetration in the cutinized cell wall and precipitation of cellular proteins (Sharma and Sharma, 1980).

The effect of 2, 4-D in callus formation using leaf explant is reported (Barna and Wakhlu, 1993) and also found effective for callus formation in other species of family Apiaceae, that is, *Coriandrum sativum* (Zee, 1981), *Dacus carota* (Halperin and Wetherell, 1964), *Foeniculum*

Table 4. Comparative account of tissue culture protocols of *Angelica* species.

Species	Explant	<i>In vitro</i> technique	Media	Response	Reference
<i>A. sinensis</i>	Embryo	Suspension cell culture	MS	Embryonic callus	Tsay and Huang (1998)
<i>A. acutiloba</i>	Shoot	Micropropagation	MS+0.01 mg/L NAA+.1 mg/L Kin	Shooting	Watanabe et al. (1998)
<i>A. panicii</i>	Rhizoma seedling	Micropropagation	MS+2 mg/L BAP MS+1 mg NAA	Multiplication Rooting	Iankova et al. (2001)
<i>A. archangelica</i>	Petiole	Organogenesis	MS+1 µm/L 2,4-D+1 µm/L BA	Callusing	Joshi (2002)
<i>A. glauca</i>	Mature leaf	Somatic embryogenesis	MS+2 µm/L NAA+2 µm BA	Somatic embryogenesis, plantlets, synthetic seeds	Present study

vulgare (Ohga et al., 1989), *Bunium persicum* (Wakhlu et al., 1990), *Heracleum candicans* (Joshi et al., 2004), and *Selinium tenuifolium* (Joshi et al., 2006).

As per somatic embryogenesis, combination of BA and NAA has been reported to induce somatic embryogenesis in *Cicer* species (Barna and Wakhlu, 1993). Previous studies have also demonstrated that a proper balance between the type and concentration of plant growth regulators at different stages of embryoid development is essential (Rangaswamy, 1986). The best responses with regards to the formation of embryos in auxins have a major impact on induction of somatic embryos in most regeneration systems (Ammirato, 1983; Raghavan, 1986).

Redenbaugh et al. (1987) observed that variables related to the encapsulated method, including type and concentration of alginate medium and method used to produce the synthetic seed, were responsible for significant variation in the conversion percentages for alfafa, carrot and celery embryos.

In the present study, better response of plantlets in sand, soil and peat moss mixture (1:1:1 ratio)

may be due to high moisture retention capacity of peat moss. In general, a high humidity and low transpiration has been considered a prerequisite for the better performance of *in vitro* raised plants during the phase of acclimatization (Kozai, 1991).

This study concluded that development of somatic embryos could be useful for mass multiplication of the species, base material for secondary metabolite production, and development of synthetic seeds production. For example synthetic seeds of the species have enough scope for cryopreservation and long term utilization. Similarly, *in vitro* production of secondary metabolite could reduce the pressure on natural habitat, however, chemical investigation need to be compared with natural stock prior to reach any conclusion.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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