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Toward a clinical research framework for collaboration among selected stakeholders in traditional herbal medical practice in seme and gem sub-locations in Nyanza province, Kenya

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Both World Health Organization (WHO) and Kenya government have recognized the role of herbal traditional medicinal practice in primary health management due to the observation that 80% of the rural communities consult traditional healers before they go for the orthodox medical services. The traditional herbal practice remains familiar and artitional, thus least developed and hence the patients do not get the best values for their input in the services. The research strategies executed by researchers have never focussed on the plight of the patients but covered phytochemical, pharmacological and no clinical evaluations. Effectively, the data so far available remain less useful than should have been to the development of herbal traditional practices. The current project thus aimed at focussing on the establishment of a research strategy that used observational studies to establish the possibility for designing a rigorous clinical trials of herbal medical practice involving participation of three herbalists, patients, one orthodox medical doctor, a plant taxonomist and a phytochemist with the objectives of proofing and validating the practice, using principles of biomedical and bioscience in Gem and Seme sublocations in Siaya and Kisumu Districts, respectively in Nyanza province, Kenya. Through the ethnobotanical field survey, the taxonomist identified 95 plant species used by the herbalists. Through literature review it was found that 100% of the plants are used by other herbalists in East Africa to treat human and animal ailments while 30% of the species had received phytochemical and pharmacological evaluations, thus validating their therapeutic values. About 10% of these species had been used in clinical studies. The observation indicate that the herbalists have reasonable potentials for management of the diseases despite their ignorance on the literature data on these plants as well as modern medical practical procedures. The inclusion of modern diagnosis of diseases by a medical doctor and medical laboratory tests improved the rate of the healing outcome by 20% when the healing rate was compared with that observed for the treatment of patients before the intervention through this project. The results so far indicate that it is practicable to implement a rigorous clinical trial in which both herbalists, patients and researchers collaborate. Such strategies shall not only give critical data for validation of herbal traditional medicinal practice but be useful for prospection for phytochemicines based on indigenous knowledge and also be used for the improvement and management of the practice.

Key words: Ethnobotany, pharmacology, phytochemistry, herbalist, patients, malaria, diarrhoea, Got Ramogi.

INTRODUCTION

In Africa, it is estimated that 75% of the rural population rely on herbal medicine for their healthcare. Herbal remedies have the advantage of being readily available, biodegradable and the process of isolation of active
ingredients is cheaper than formulating and producing synthetic drugs (Ampofo and Johnson-Romauld, 1978). The benefit of this observation has not been realized in Africa because the research strategies used in the study of traditional medicinal practices have not been focused on the objectives which are meant to support the practitioners and the patients. The research have been on phytochemical and pharmacological evaluations without any on clinical trials and toxicological evaluations including those on standardization of the herbal remedies as well as on modern medical diagnostic practices. There have never been attempts at training the traditional practitioners and integration of medical skills in the practice including the good harvesting, manufacturing and administration of the remedies. The research paradigms neglected the integration of clinical and laboratory diagnostic techniques in the traditional practice. The ethnomedical, ethnomedicinal, phytochemical and pharmacological evaluations done in Kenya have neglected the patients’ feelings, attitudes, benefits and welfare (Sidiga 1995; Kokwaro, 1994; Jeruto, 2008; Gakunju, 1995; Ostrom, 2008) including the evaluation of the practitioners’ practice.

Patient-centred study by Alamo (2002) was found more effective than the usual consultant-centred evaluation of effectiveness of treatment of musculoskeletal chronic pain using Chinese herbal remedies. It is also observed that there is no published information in Kenya that evaluates the reliability, efficacy, safety and validity of the traditional medical practices’ performance using an approach recommended by Lewis and Elvin-Lewis (1994) and Steven and Jeffrey (2003) and participatory research approach developed for Canadian overseas projects (Found, 1995) in which all stakeholders are participants in all stages of the research.

In 1980, Clinicians working in cooperation with Chinese medicine practitioners using aqueous extracts of ten Chinese medical herbs in London treated children suffering from severe atopic eczema. In a double blind placebo controlled trial, using 37 young patients, the research proved that treatment was achieved only if all the ten plants were used (Williamson, 2001). Other challenges which must be considered in such research paradigms are ethical issues and positive attitudinal changes. The intellectual property rights, indigenous knowledge, benefit sharing, efficacy and safety must be factored into the research activities. The change of attitude and building trust among the research team, the herbalists and the patients must be undertaken so as to obtain maximum benefits to all the stakeholders participating in this project.

It is believed that these strategies must be integrated in the practice with the objectives of validation and value addition to the practice. Such efforts shall aid rational decisions on the formulation of policies and their implementation for the control of herbal medical practices and therefore health management and socio-economic development while exploiting the biodiversity in a sustain-

able manner.

The foregoing information directed the research strategies in which rural rapid appraisal and participatory research method to test the suitability of the strategy that was employed to conduct the pilot project herein described. Thus the team made up of a forester, plant taxonomist, phytochemist and a medical doctor organized a meeting with three herbalists and their patients where questionnaires and discussions were conducted to obtain information on identification of the plants, disease diagnosis and treatments. The literature on the ethnomedical, ethnomedicinal, phytochemical and pharmacological data were collected and analysed so as to validate the use of the plants in treatment of the diseases by the herbalists. The information so far obtained are found useful for the design and future implementation of observational and clinical trials in herbal medical practice in Kenya that would lead to value addition to the traditional medicinal practice.

MATERIALS AND METHODS

The project setting

The recruitment of research team and patients

The herbalists and patients were recruited from Seme and Gem sub-locations of Kisumu and Siaya districts. A female herbalist was recruited from Gem sub-location while two male ones were recruited from Seme sub-location. The criteria for the selection of the herbalists was based on their education, age, experience and our contact with them in another project on the phytochemical and pharmacological evaluations for sustainable exploitation of Toddalia asiatica (Orwa et al., 2008). These herbalists recruited the patients whom they treated under the supervision of the medical doctor. The taxonomist was recruited on the basis of his experience in the identification of the plants in the fields. The recruitment of patients were done in Seme sub-location in Kombewa division in Kisumu District and South Gem sub location in Gem Division in Siaya District in Nyanza province in Kenya. Seme sub-location has no hospital but four, six and one health centres, dispensaries and clinic nursing home, respectively serving a population of about 50,000 people (GOK, 1996). This sub-location is in Siaya District where in 1990 to 1992, top ten causes of morbidity due to diseases were malaria, acute respiratory infection, skin infection, diarrhoeal diseases, intestinal worms, urinary tract infection, eye infection, rheumatism, ear infection and gonorrhoea with disease prevalence of 42, 23.8, 8.5, 6.7, 3.9, 3.2, 2.6, 1.7, 1.6 and 1.5%, respectively (GOK, 1993).

South Gem sub location is served by three and six dispensaries and health centres, respectively and has a population of 70,000 people. The disease prevalence is probably similar to the situation in Seme sub location, Siaya, Bondo and Kisumu towns have each one district hospital which is supposed to serve the two sub locations which are however in the rural areas, approximately 50 kilometres away from these towns. These rural areas have poor road networks and the health services due to poor personnel and drug availabilities as well as few all-weather roads. This indicates that the most immediate health services are accessed through traditional healers including herbalists. These factors were considered during the recruitment of the patients at random. In Seme, the herbalists were stationed at Kondik Trading centre while in Gem the recruitment was done at Ebusakami Primary School.
where each reporting patient was asked to sign the treatment agreement form after the objectives of the project was discussed with them.

The discussion on the nature of herbal medicinal treatment they were to get was adequately explained to them before diagnosis and treatment processes.

Workshop for the herbalists and the patients

The recruited patients and the herbalists were called for a three day workshop at Kondik Trading centre at which the three herbalists and fifty recruited patients were taught by the facilitators on the objectives, principles, implementation and benefits of the project to each of the stakeholders as well as the nature and issues on good herbal medical practices. The herbalists were trained on simple taxonomic, preservation and preparation techniques of herbal products. They were also given lectures on some simple methods of diagnosing and identification of the common diseases including clinical management of patients. Further, the herbalists were given lectures on the general values of research to the herbal medicinal practice with respect to the government health policies on their practice.

Questionnaires were administered to the herbalists to establish the value of the research activity compared to their earlier practice. The herbalists were also given a three hour discussion in the standardisation and measurements introduced in preparation of herbal remedies as well as diagnosis of the diseases. The patients were taught the knowledge of the common diseases they usually contact in the areas. There was a lecture on the practice of herbal medicinal practice which was meant to create awareness and attitudinal change towards misconceptions held on herbalists, herbal remedies and the practice in the rural societies and expert communities. There was a general discussion session in which stakeholders were given time to give their reflection on the herbal medical practice. Questionnaires were administered to the patients to assess their knowledge and attitudes towards the herbal medical practice they might have attended before and compared to the current one.

Identification of the herbal plants

The research team visited the herbalists who took the team around to observe the plants in their habitats for taxonomic identification. The plants which were not found at Got Ramogi, Seme and Gern sub-locations and within the vicinity of the area were collected and given to the taxonomist for identification. The herbalists were taught how to preserve the specimen in a box supplied by the project. The plant specimen was kept in paper by the herbalists for future identification of the plants for collection and processing.

Literature study of the ethnobotanical, pharmacological and phytochemical information

The databases such as NAPRALERT, AFLORA (AFLORA (2008)), MEDFLOW, PHARMEL 2 and PRELUDE (Fernsworth, 1994), textbooks, journals, conference proceedings and the internet were sourced for botanical ethnobotanical, pharmacological, toxicological and phytochemical information available in the literature about the identified plant species. The information were analysed to validate the medicinal uses of the plants by the herbalists and also to aid preparation of the remedies. The data was tabulated and analysed to guide the standardization of the preparation of the herbal products for use by the herbalists and for formulation of future research agenda.

Preparation of the herbal remedies

The remedies were standardized by weighing and measurement of the volumes of water used in the preparation of decoctions from wet plant parts. The wet plant parts were shredded by chaff-cutter before grinding them by a plate mill. The tincture of the wet plants parts were made in a mixture of 2:1 ratio of water: whisky over a period of three days. The dry plant parts were dried under shade and burned to ash. The other plant parts were similarly dried and ground using a hammer mill to fine powders which were then mixed in ratios by weights as directed by the herbalists. The weighed amounts of dry plants were put in definite ratios to be decoted in water as directed by the herbalists. The decoctions were measured by graduated cups when dispensing to the patients. Some plants were placed into a saucepan and burned to ash to be used for treatment of wounds whereas some were dried and rolled into paper to be smoked for the treatment of asthmatic cases. Both powders and ashes were standardized by passing them through a sieve. The water extracts were filtered to obtain the suspended particles and dissolved compounds as well as pH. The density of the filtrate was obtained for standardization. The water extracts were then administered to the patients in doses advised by the herbalists. The decoctions powders and ashes were labelled by the coded names and the diseases for which they are indicated.

Standardization of the prepared remedies

The decoctions of both wet and dry plant materials were done by weighing of the wet and dry plants before putting them into measured water whose final volume was measured after boiling. The mass of both suspended particles and the dissolved compounds were measured so as to know how much substance was given to the patients in a measured volume. The pH of the decoction was also taken.

The particle sizes of the ground materials were standardized by the size of the sieve of the grinder as well as sieves for 5 to 10 micron particles. The ash which was standardized by a sieve was suspended in distilled water so as to take its pH. The rolled powdered plant material was weighed and put into a paper of known size. The decoctions were filtered and density measured then freeze dried and dissolved in a mixture of 1:1 ratio of methanol and chloroform and analysed by thin layer chromatographic technique. The number of spots identified by ultra-violet lamp and 50% sulphuric acid were used for standardisation.

Diagnosis of diseases

The patients were recruited by the herbalists and taken to Kondik trading centre of Ebusakami Primary School where they were diagnosed by both the herbalists and the medical doctor at the beginning of the project to obtain pre-treatment diagnosis. The herbalists listened to and observed the medical doctors’ clinical observation of patients. The same activity was done by the medical doctor as the herbalists interviewed the patients. At the end of the clinical interviews both the herbalists and the doctor harmonised the diagnosis for each patient before and after each treatment. The patients whose diagnosis required laboratory tests were taken either to Kombewa Health Centre or Maseno Hospital of Anglican Church or Kisumu District Hospital. The project paid for the costs of the tests. After the diagnosis the herbalists administered the herbal preparations. The patients were given appointments to see the herbalists every day in the evening to review their cases. Once the patients indicated that they were feeling well then they were reviewed by the medical doctor and the patients whose cases required post-treatment laboratory tests were sent to the laboratories for confirmation of successful treatment.
RESULTS AND DISCUSSION

The species of plants used by the three herbalists

In total, 95 plant species found in 41 families were identified with frequency of occurrence of 57, 54 and 51 in Got ramogi, Seme and Gem, respectively (Table 1). Only three species were obtained from Eldoret and Kisumu towns through vendors. The plants collected from Got ramogi were purchased by the project for the herbalists at half dollar per bundle of 2 kg of wet plants. The plants collected at Gem and Seme were not purchased since they were from the herbalist’s home gardens or live fences in Seme and Gem or conservation sites in Seme. The percent of the plant species collected from home gardens and conservation sites were 80 and 20%, respectively. The conservation site is owned by Miguye conservation group.

The biodiversity was represented by the 12 families of the plants with the number of species indicated in the brackets: Compositae (13), Labiatae (10), Euphorbiaceae (6), Solanaceae (4), Caesalpinioidea (4), Anacardiaceae (3), Bignonieae (3), Cucurbitaceae (3), Meliaceae (2), Myrtaceae (2), Malvaceae (2), Convolvulaceae (2) and Verbenaceae (2), whereas the following 26 families were each represented by one species: Rutaceae, Simaromraceae, Papilinioideae, Gutiferae, Rosaceae, Urticaeae, Hypoxidaceae, Leguminosae, Combretaceae, Acanthaceae, Menispermaeae, Caricaceae, Rubiaceae, Papilinioideae, Mimosoideae, Phytolaccaeae, Musaceae, Vitaceae, Liliaceae, Amanthaceae, Cappressaceae, Moraceae, Umbelliferae, Asclepiadaceae, Apocynaceae and Astraraceae. The popularity of the use of the families are corroborated by the observation recorded by Kasonja, et al. (1997) in their study of the ethnoveterinary plants in the Lake Victoria region. A moss plant species was not identified.

The frequencies of the plant parts used were given in brackets as seeds (1), flowers (6), fruits (30), root bark (39), whole (44), leaves (52) and stem bark (53) (Table 1). Literature review using databases such as NAPRALERT, AFLORA, PROTA, MEDFLORA, PHARML 2 and PRELUDE including textbooks and journals (Farnsworth, 1994; Kokwaro, 1994; Hans, 1996) revealed a lot of corroborations on the types of diseases treated by other herbalists in several societies in East Africa as indicated in Table 1. The herbalists treated 16 diseases using a multiple of plants prepared from fresh or dried materials in the form of decoctions or tinctures or burnt materials and administered orally, smoked or applied on affected skins of the patients. The literature survey indicated that the plants were ethnomedically indicated for a wider range of diseases (Table 1). The 60 and 32% of the species of the plants included in the study had received pharmacological and phytochemical evaluations which were used to validate some of the treatment claims given by three herbalists and those from other ethnic backgrounds (Tables 1 and 2). The plants in Table 2 were used by one of the herbalists in making the decoction called a multipurpose remedy that was orally given patients suffering from several ailments (diseases 12 to 19) in Table 3. It was also encouraging to note that Centella asiatica, Withania somnifera, Spilanthes mauritiana, Achyranthes aspera, Mongifera indica, Psidium guajava, Lantana trifolia and Euphorbia hirta which were used by the herbalists in this project had been used in clinical trials. A balm of C. asiatica (0.03%) and S. mauritiana (0.03%) applied topically in the forehead of patients reduced the symptoms of migraine headaches 19 min after applications (Patent No. 200610658122), while essential oils from A. aspera were used in observational studies of asthmatic patients with positive results.

The clinical trial studies have been conducted using P. guajava (Xavier et al., 2006), E. hirta, M. indica and L. trifolia for treatment of patients suffering from diarrhoea, amoebic dysentery, liver ailment and pulmonary tuberculosis, respectively with reasonable results. Clinical trials with the aqueous extracts of leaves of A. conyzoides (Kamboj et al., 2008) on patients suffering from arthrosis indicated that 66% of them experienced the analgesic effects while 24% of them had improved articulation and mobility without side effects. Clinical trials on human volunteers were conducted with 4% endod (Phytolacca dodecandra) ointment against dermatomyces and found to be effective (Jassim, 2003). Artex Mendar, a standardized multiplant Ayurvedic drug compost of W. somnifera, B. serrata, Zinger officinalae and Curcuma longa was used in controlled clinical trials on 358 patients suffering from symptomatic osteoarthritis of the knees. The results indicated reasonable efficacy and safety over a period of 32 weeks (Chopra et al., 2004; Srivansana et al., 2007). The clinical trials give evidence that the traditional herbal medical practice can be an avenue for discovery of herbal remedies which can be used to treat the people who may not be able to access government health facilities.

The physico-chemical parameters

The sizes of the fresh materials were cut with a chuff cutter to about 2 to 4 cm and 5 to 6 cm in length and width, respectively before grinding them in a plate mill. Both juice and the solid residues were transferred to the saucepan for boiling. The sample was then strained by tea strainer and the residues thrown away. The decoction was the set for treatment. A portion was then taken for measurement of physico-chemical parameters. The dry plant materials were milled by a hammer mill to obtain 5 to 10 micron particles whose sizes were confirmed by sieve measuring device. The powders were infused in boiling water and filtered. A portion of the decoctions from both fresh and dry plant materials were used to obtain physico-chemical parameters. The density was about 0.9 g/ml while the pH range was 6.0 to 7.9. The undissolved
Table 1. The species of the plants used by three herbalists.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Family</th>
<th>Place of collection</th>
<th>A: Parts used</th>
<th>B: Diseases indicated by the herbalists</th>
<th>C: Diseases indicated in the literature (Kokwaro, 1994)</th>
<th>D: Pharmacological evaluations</th>
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Table 1. Contd.

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<th>Plant Name</th>
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<th>Type</th>
<th>Accessions</th>
<th>Compounds</th>
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<td>Anadenanthera viridis</td>
<td>Raubasine, reserpine</td>
<td>Anti-inflammatory, antioxidant, analgesic, antiallergic, antidiarrheal, antiasthmatic, antidepressant, antiplasmolytic, antihistamine, antimutagenic, and immunosuppressive properties; 6. Anti-inflammatory, antioxidant, analgesic, antiallergic, antidiarrheal, antiasthmatic, antidepressant, antiplasmolytic, antihistamine, antimutagenic, and immunosuppressive properties; 7. Antifertility.</td>
</tr>
</tbody>
</table>

Table 2. The phytochemical and pharmacological evaluations reported on the plants used by the herbalists in this project.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Compounds</th>
<th>Pharmacological properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catharanthus roseus</td>
<td>Vincristin, vinblastin</td>
<td>Anticancer activity and used for treatment of cancer (Daniel, 2006).</td>
</tr>
<tr>
<td></td>
<td>Raubasine, reserpine,serpentine</td>
<td>Antifibroidal and antihypertensive activity.</td>
</tr>
<tr>
<td>Plant Name</td>
<td>Constituents</td>
<td>Activity</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Table 2. Contd.</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Contd.

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Chemical Compounds</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Garcinia buchananii</em></td>
<td>3-geranyl-2,4,6-trihydroxy-benzo-phenone, 1,3,5,7-tetrahydroxy-8-isoprenyl-xanthone, 1,3,5-trihydroxy-8-isoprenyl xanthone, 3-geranyl-2,4,6-trihydroxybenzophenone and butinolic acid</td>
<td>Activities against <em>Candida albicans</em> and <em>Staphylococcus aureus</em> (Han et al., 2006).</td>
</tr>
<tr>
<td><em>Vernonia amygdalina</em></td>
<td>Luteolin, luteolin-7-O-beta-glucoside, vernadalin, vernonioside B1, vernonioB1 and vernoamygdalin.</td>
<td>Antioxydant, antiplasmodia, antischisotosomial, antileishmonial and antihepatotoxic properties. (Iwalokun et al., 2006)</td>
</tr>
<tr>
<td><em>Entada abyssinica</em></td>
<td>Isokolavenol and it glycoside, kolavic acid</td>
<td>Anidiabetic, antitypanosomal activities (Nyasse et al., 2004a, b)</td>
</tr>
<tr>
<td><em>Rubia cordifolia</em></td>
<td>Rubiadin, emodin, physion, luteolin, quercetin cyclic peptide, 1-hydroxy-2-quinone, 1,4-dihydroxy-2-methyl-5-methoxyanthraquinone, 1,3-dimethoxy-2-carboxyanthraquinone, 6-methoxy geniposidic acid, manjistin, garasin, alizarin, rubiprasin and rubiarbonal</td>
<td>Antibacterial, anti-inflammatory, antiplatelet, hepatoprotective, antioxidant, anti-cancer, antihepatitic, antiallergic, anti-ulcer, antidiabetic properties (Basu et al., 2005).</td>
</tr>
<tr>
<td><em>Croton microstachys</em></td>
<td>Crotopoxide, neo clerodan-5,10-en-19, 6-beta-20-dienolide, 3-alpha-19-dihydroxytrachylopane, 3-alpha-18,19-trihydroxytrachylopane</td>
<td>Antitumor activity (Daniel, 2006)</td>
</tr>
<tr>
<td><em>Markhamialutea</em></td>
<td>Luteoside A, B, &amp; C, verbascoside and isoverbascoside</td>
<td>Anti viral activity against respiratory syncytial virus (Kerman et al., 1998)</td>
</tr>
<tr>
<td><em>Spilanthes mauritania</em></td>
<td>N-isobutyl decadienamide</td>
<td>Antiplasmodial activity (Jondiko, 1986; Weenen et al., 1990)</td>
</tr>
<tr>
<td><em>Carica papaya</em></td>
<td>Carpain and papain</td>
<td>Cardiotonic and antihelmimthic activities (Daniel, 2006)</td>
</tr>
<tr>
<td><em>Bryonia dioica</em></td>
<td>Bryodins 1 and 2</td>
<td>Anticancer activity (Siegall et al., 1994)</td>
</tr>
<tr>
<td><em>Ageratum conyzoides</em></td>
<td>Essentialols, 6-(1-hydroxymethyl)-7, 8-dimethoxy chromene, 6-hydroxy-7,8-dimethoxy-2,2-dimethylchromene, coumarins, isoflavones.</td>
<td>Antibacterial activity (Kamboj et al., 2008)</td>
</tr>
<tr>
<td><em>Withania somnifera</em></td>
<td>Dimeric thiowithanolide, withanoside D, sominine, withaferin A, monomeric glycoprotein(28KDa)</td>
<td>Anticancer activity and antibacterial activities (Subbaraju et al., 2006, Sirinivasan et al., 2007, Kobuyama et al., 2006)</td>
</tr>
<tr>
<td><em>Ajugar remota</em></td>
<td>Ajugarin1, Ajugarin 2, ergostenol-5,8-Endoperoxide</td>
<td>Antiplasmodial activity, anti-mycobacterium tuberculosis (Manguro et al., 2005).</td>
</tr>
<tr>
<td><em>Cassia spectabilis</em></td>
<td>3-O-acetylspectalin, (−)-7-hydroxy-spectalin, iso-6-spectalin</td>
<td>Antifungal and antinoreceptive anti activities (Viegas et al., 2008)</td>
</tr>
<tr>
<td><em>Datura stramonium</em></td>
<td>Hyoscine, atropine and hyoscyamine, Stimulant, sedative, hypotetic, antiacetylcholinestrase</td>
<td>Antispasmodic (Daniel, 2006)</td>
</tr>
</tbody>
</table>
Table 3. The treatment of patients and administration of the prepared herbal remedies as practised by the herbalists in this project.

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Number of patients</th>
<th>Plant used (part used)</th>
<th>Preparation of herbal remedies</th>
<th>Administration of herbal remedies and outcome of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial asthma</td>
<td>4</td>
<td>Leonotis molissima (leaf), Terminalia brownii (bark), Bryonia dioica (leaf), Datura stromonium (seed)</td>
<td>Ten grammes of equal ratios of L. molissima, T. brownii and B. dioica powders was infused in 150mls of water for 15 minutes and filtered. Twenty drops of tincture of D. stromonium in whisky steeped for seven days were put into 70mls of water.</td>
<td>The patient drank 150 mls of infusion and 150 mls of diluted tincture daily for two weeks. Clinical symptoms of three patients subsided while one patient did not heal.</td>
</tr>
<tr>
<td>Bronchial pneumonia</td>
<td>3</td>
<td>Melia azadirach (leaf), Erythrina excelsa (bark)</td>
<td>Twenty grammes of equal ratio of powders of the plants were boiled in 150 ml of water for 15 minutes and filtered.</td>
<td>The patient was orally given 75 mls of the decoction three times daily for 8 days. All patients healed.</td>
</tr>
<tr>
<td>Malaria</td>
<td>4</td>
<td>Erythrina abyssinica (stem bark), Toddalia asiatica (root bark), Microglossa pyrifolia (leaf)</td>
<td>Ten grammes of equal amounts of the powders of the plants are infused in 200 mls of boiling water for 20 min and filtered.</td>
<td>The patient orally drank 100 mls of the infusion three times daily for two weeks. All patients had no clinical symptoms and parasitamia.</td>
</tr>
<tr>
<td>Malaria</td>
<td>3</td>
<td>Erythrina abyssinica (stem bark), Toddalia asiatica (root bark), Kigelia africana (fruit), Ficus lutea (stem bark)</td>
<td>One kilogram in equal amounts of fresh plant materials were cut into small pieces and ground by a plate mill and boiled in one litre of water for one hour then filtered.</td>
<td>The patients were given 150 mls of the decoction three times daily for two weeks. Two patients showed no clinical symptoms and parasitamia while one patient had persistent symptoms of both.</td>
</tr>
<tr>
<td>Intestinal worms</td>
<td>3</td>
<td>Albizia coriaria (stem bark)</td>
<td>Ten grammes of the powder was infused in 150 mls of warm water for ten minutes and filtered.</td>
<td>The patients were orally given 75 mls three times daily for two weeks. Two patients tested negative for casts of worms with disappearance of clinical symptoms while patient did not heal.</td>
</tr>
<tr>
<td>Intestinal swelling nodes with headache and dizziness</td>
<td>4</td>
<td>Viscum album (whole plant), Taraxacum officinale (whole plant)</td>
<td>Twenty grammes of equal amounts of the powdered plants were decocted in boiling 600 mls of water for twenty minutes and filtered.</td>
<td>The patient drank 150 mls of decoction twice daily for three weeks. Clinical symptoms in two patients subsided while one did not heal.</td>
</tr>
<tr>
<td>Allergy and fungal skin infection</td>
<td>4</td>
<td>Urtica dioica (leaf), Senna didymobotrya (leaf), Leonotis molissima (whole plant), Ricinus communis (seed, leaf)</td>
<td>Ten grammes of equal amounts of the leaf powders of three plants together with that whole L. molissima were decocted in 150mls of boiling water for ten minutes and filtered. An ointment of five grammes of the leaf powder of S. didymobotrya and seeds of R. communis in equal amounts was made in 10mls of the oil of R. communis.</td>
<td>The patient drank 75 mls of the decoction three times daily for two weeks. The ointment was applied on affected skin twice daily for three weeks. The clinical symptoms in three patients disappeared while one did not heal.</td>
</tr>
</tbody>
</table>
### Table 3. Contd.

<table>
<thead>
<tr>
<th>Condition</th>
<th>No.</th>
<th>Plants Used</th>
<th>Preparation</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectic ulcer</td>
<td>6</td>
<td><em>Datura stromonium</em> (leaf), <em>Centella asiatica</em> (whole), <em>Aspilia mosambiensis</em> (whole), <em>Withania somnifera</em> (leaf), <em>Tussilago vulgaris</em> (whole), <em>Moss sp.</em> (whole)</td>
<td>A tincture of 10 grammes of macerated fresh leaves of <em>D. stromonium</em> was made in 20mls of whisky for 7 days and strained. Twenty grammes of the powders in equal amounts C. asiatica, <em>baccilicum</em>, W. somnifera, <em>T. vulgaris</em>, Moss sp. and <em>A. mosambiensis</em> were boiled in 150mls of water for 20 minutes and filtered.</td>
<td>The patient was orally given 75 mls of the decoction in which 20 drops of tincture were added three times daily for three weeks. The clinical symptoms and barium meal tests showed improvement in all patients.</td>
</tr>
<tr>
<td>Bronchial asthma</td>
<td>4</td>
<td><em>Carica papaya</em> (leaf), <em>Euphorbia hirta</em> (whole), <em>Eucalyptus citrodora</em> (leaf), <em>Maribium vulgaris</em> (leaf), <em>Datura stromonium</em> (seed, leaf)</td>
<td>Half gramme of equal amounts of powdered leaves of four plants and whole <em>E. hirta</em> was rolled as a cigarette in plain paper. A decoction of twenty grammes of the powder of the plants in equal amounts were made in 150mls of boiling water for twenty minutes and filtered. A tincture of 20 grammes of equal amounts of fresh leaves of the plants together with one gramme of ground seeds of <em>D. stromonium</em> were steeped in 100 mls of 20% surgical spirit for seven days then strained.</td>
<td>The patient smoked the cigarette and drank the 150 mls of the decoction containing 12 drops of tincture three times daily for three weeks. The clinical symptoms subsided in three patients while one patient did not heal.</td>
</tr>
<tr>
<td>Amoebic dysentery</td>
<td>10</td>
<td><em>Carica papaya</em> (leaf), <em>Euphorbia hirta</em> (whole), <em>Harrisonia abyssinica</em> (stem bark), <em>Cyphostemma nodiglandulosa</em> (corn. leaf), <em>Ajuga remota</em> (leaf)</td>
<td>Twenty grammes of equal amounts of each part of the plants were decocted in 150 mls of boiling water for 15 min and filtered.</td>
<td>The patients drank 150 mls three times daily for three weeks. The clinical symptoms and laboratory evidence indicated that eight patients were treated from the disease while two did not.</td>
</tr>
<tr>
<td>Facial skin allergy due to cosmetics</td>
<td>3</td>
<td><em>Coryza sumatrensis</em> (leaf), <em>Bidentis pilosa</em> (leaf), <em>Aloe kedogensis</em> (leaf), <em>Bredelia micrantha</em> (leaf), <em>Ageratum conyzoides</em> (leaf), <em>Cassia spectabilis</em> (leaf)</td>
<td>A decoction of three kilogrammes of equal amounts of fresh plants were made in two litres of boiling water for one hour and strained. The gel from fresh leaves of <em>A. kedogensis</em> was expressed manually. One kilogramme of leaves of <em>A. spectabilis</em> was burned and ash kept for use.</td>
<td>The patients drank 75 mls of the decoction and applied the gel on affected skin twice daily for three weeks. The clinical symptoms subsided in all patients.</td>
</tr>
<tr>
<td>Infected wounds on legs</td>
<td>3</td>
<td>The plants were the same as the ones for facial allergy due to cosmetics.</td>
<td>The preparation was the same as the one for the case for allergy due to cosmetics.</td>
<td>The patient drunk 150 mls of the decoction and washed the wounds with it and also applied the gel and the ash on the affected skin three times daily for three weeks. All patients healed as evidenced from clinical observation.</td>
</tr>
<tr>
<td>Fungal infection on the scalp</td>
<td>10</td>
<td>The plants were the same as those used for treatment of allergic case.</td>
<td>The preparations were same as the ones used in the treatment of allergic case.</td>
<td>The patients orally drunk 150 mls of the decoction, washed the surface with it and applied the gel on the surface three times daily for three weeks. All patients did not show clinical symptoms of the skin disease.</td>
</tr>
</tbody>
</table>
### Table 3. Contd.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number</th>
<th>Description</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric hyper acidity and arthritis</td>
<td>4</td>
<td>The stem bark and leaves of the plants in Table 2 were mixed, ground and boiled in water</td>
<td>Ten kilograms of equal amounts of fresh parts of the plants were chopped into small pieces and ground by a plate mill then boiled in ten litres of water and filtered</td>
<td>The patient drank 150 mls of the concoction three times daily for three weeks. Three patients showed no clinical symptoms while one did not heal</td>
</tr>
<tr>
<td>Chronic menorrhagia</td>
<td>5</td>
<td>The plants were the same as those used for treatment of gastric hyper acidity and arthritis.</td>
<td>The concoction was the one used for treatment of patients suffering from gastric hyper acidity and arthritis</td>
<td>Administration was the same as that used for treating gastric hyper acidity and arthritis. Four patients indicated no clinical symptoms of the disease while one did not heal</td>
</tr>
<tr>
<td>Elephantiasis</td>
<td>2</td>
<td>The plants were as those used in the treatment of chronic menorrhagia.</td>
<td>The concoction was the same as that used for the treatment of chronic menorrhagia</td>
<td>The patient drank 150 mls of the concoction and cleaned the lesions with it three times daily for one month. There was no improvement</td>
</tr>
<tr>
<td>Herpes zoster</td>
<td>2</td>
<td>The plants were the same those used for treatment of chronic menorrhagia.</td>
<td>The concoction was the same as that used for the treatment of chronic menorrhagia</td>
<td>The patient drank 150 mls of the concoction and applied the ash from <em>A. spectabilis</em> on the wound three times daily for three weeks. The wound and pain subsided as indicated by clinical observation and the two patient’s response</td>
</tr>
</tbody>
</table>

Substances in the decoctions ranged between 0.0002 to 0.0003 g per ml whereas the dissolved substances were between 0.0002 to 0.0004 g per ml. The thin layer chromatographic analysis indicated that most of the extracts of the decoctions had 5 to 10 spots. The parameters indicated that the decoctions contained both insoluble and soluble substances or compounds which could be responsible for the therapeutic properties of the herbal drugs. These parameters can be tentatively used for standardization of the herbal drugs as well as the quantification of the doses.

**Clinical and observational evaluations**

The patient and herbalist focused approach in ethnomedicinal studies led to the identification of the listed 95 plant species in Table 1 and the diseases indicated for them by the herbalists in Table 3. The literature survey on ethnobotanical uses of these plants revealed very widespread use of these plants thus validating their use by the chosen herbalists in the project. Further phytochemical and pharmacological literature survey confirms our conviction that despite low educational capacity and poor access of information to the herbalists, the traditional practice has a lot to guide the discovery of potential drugs for treatment of the diseases.

Table 3 indicates that half of the plants used by the herbalists had received reasonable pharmacological and phytochemical evaluations thus further validate the medicinal practice by these herbalists. The incooperation of the medical doctor in the...
project also indicated that the diagnostic capability of the herbalists did not show wide variation. There was qualitatively close agreement between the diagnosis given by the herbalist and that given by the doctor in 80% of the diseases. Such diagnoses were confirmed by both pre-treatment and post-treatment clinical laboratory tests where it was applicable. The percent healings were as indicated by bracket for each skin diseases (95%), bronchial and asthmatic pneumonia (82%), intestinal disease and headache (70%), dysentery (80%), chronic monorrhagia (80), gastric acidity and arthritis (75%), pectic ulcer (100%), intestinal worms (66%), herpes zoster (100%) and elephantiasis (0%). The medical doctors’ observation and laboratory diagnosis confirmed the healing rates. The questionnaires administered to the herbalists with respect to healing rates reported for same period before this intervention indicated an improvement of 10 to 20% healing rates for most of the diseases. The details of the plants species, preparation and administration of the herbal medicines are indicated Table 3. The pharmacological, phytochemical and clinical trials attributed to the species are believed to contribute to the high healing rates between 66 to 100% except for elephantiasis for which there was no healing. It is important to note that all the herbal remedies were made of multiple plants except for treatment of intestinal worms in which only one plant was used (Table 3). These plants were indicated for treatment of multiple diseases. For example, percentages of species indicated for 5, 8, 7, 6 and 4 diseases were 50, 5, 2, 3 and 1%, respectively (Table 1). A survey of ethnomedical literature revealed that other herbalists elsewhere in East Africa employ polyherbal and multi-disease treatment approach. For example, 20% of the species are indicated for at least 8 diseases. Fifty percent of the plant species identified by the herbalists were indicated for nearly fifty diseases thus confirming the importance of these plants in disease management.

The pharmacological evaluations using either in vitro or in vivo methods indicated that 45% of the solvent extracts of the species had antifungal, antibacterial and antiviral activities including physiological properties which validate the therapeutic or healing values observed in this project (Tables 1 and 2). The physiological properties such as anti-inflammatory, antioxidant, analgesic, hyperglycaemic and hepatoprotective activities of the crude extracts and the isolated compounds lend credence to the therapeutic observations in this project and thus confirms the hypothesis that the plants used by the herbalists have chemical, protective and medicinal principles.

Table 2 contains 31 plant species and 80% of which are used by one of the herbalist in the preparation of the multipurpose herbal remedies orally given to patients suffering from several ailments. These plants had received phytochemical and pharmacological evaluations leading to isolation and structural elucidation of antifungal, antibacterial and antiviral as well as antimalarial properties. The ethnomedical, ethnopharmacological and phytochemical data not only validated the traditional medical practice but opens avenue for the next research agenda as well as clinical trials which would lead to value addition to the practice and proper health management.

AKNOWLEDGEMENTS

I sincerely thank the Regional Programme for Sustainable Use of Dryland Biodiversity for the research grant as well as Dr. Jeff Odera for his support during the research activities. My sincere thanks are due to Mr. Simon Mathenge who identified the plants. The three herbalists; Mrs Mary Muga, Mr. Okaka and Mr. Obunga must be profusely thanked for their contribution in showing us the plants, recruiting the patients and the treatment they gave to the patients. I must thank Dr. Mannase Onyimbi who kindly agreed to participate in the project as an orthodox doctor against the medical ethics as an observer but also to help us diagnose the diseases. Finally, I must thank the patients and the medical laboratory technicians who took part in the project.

REFERENCES


Full Length Research Paper

The medicinal characteristics of alcohol-extraction-water-precipitation fraction from *Swertia mussotii* Franch.

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The alcohol-extraction-water-precipitation fraction of *Swertia mussotii* Franch. (SME-d) had been proved to be hepatoprotective without toxicity in previous report. In this article, high performance liquid chromatography (HPLC), rat experiment and P450 model tests were employed for studying the pharmacology characteristics of SME-d. The results showed that the contents of sweroside, swertiamarin, mangiferin, gentiopicroside, and isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively. SME-d could reduce the CCl₄-induced exaltation of alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), total bile acid (TBA) significantly in rat, and the protective activity showed dose-dependent in 0.9 and 1.8 g/kg body weight (BW). The hepatoprotective activity of SME-d was different to positive drug bifendate, which only did on ALT value significantly. Bifendate could inhibit 66.17 ± 2.12% of CYP3A4 activity, while SME-d showed 99.0 ± 0.267% reductions on CYP1A2. The different medicinal characteristics of SME-d to bifendate, which are used widely to cure hepatitis in china, can give more choices for hepatitis.

Key words: *Swertia mussotii* Franch, the alcohol-extraction-water-precipitation fraction, pharmacology characteristic, hepatoprotective activity.

INTRODUCTION

Liver is an important organism for the metabolism and detoxification of various components entering into the body, and is hurt usually by the toxins and drugs, viral infections (Hepatitis A, B, C, D, etc.) and microbial infections (Sharma and Ahuja, 1997). Hepatitis is a big challenge to the modern medicine always. Plant-based traditional medicines were widely and successfully used in the treatment of liver disorders, for example, *Picrorhiza kurroa* (Chander et al., 1992), *Phyllanthus emblica* (Gulati et al., 1995), *Silybum marianum* (Flora et al., 1998). Bifendate, coming from herb *Schisandra chinesis*, had been the common drug for hepatitis in China (Pan et al., 2006).

*Swertia mussotii* Franch., referred to as “Zang Yin Chen” in Chinese, is a biennial herb of the family Gentianaceae that has been widely used in Tibetan folk medicine. *S. mussotii* is often used to remedy diseases in liver (Yang, 1991). Inventing a new medicine for hepatitis is always a big objective and challenge to plant chemist from *S. mussotii* Franch..

The alcohol-extraction-water-precipitation fraction from *S. mussotii* Franch. (SME-d) had been proved to be hepatoprotective without obvious toxicity in mice (Lv et al., 2010). If we wanted to devise a new medicine in china, 50% composition should be identified for injection medicine, its function should be proved in rat, the dose relationship should be illustrated, and the effects on P450 activity should be clarified. So high performance liquid
chromatography (HPLC), hepatoprotection evaluation in rat and P450 model test were employed for answering these questions.

MATERIALS AND METHODS

The preparation of SME-d

The whole plant of S. mussotii Franch. (SM) was collected from Sichuan province, China, and at the full-blooming stage in July, 2008. It was authenticated by the Centre of Tibetan Medicine, Northwest Institute of Plateau Biology, Chinese Academy of Sciences. 1 g crude SM was subjected to 10 ml ethanol (75% v/v) in hot water bath for three times, and the ethanol was removed by distillation under reduced pressure. Then the ethanol eluctate was dissolved in distilled water (1:8 v/v) for 24 h, and centrifuged (8000 rpm/min) for 10 min. At last, the sedimentation was taken as SME-d.

Chemicals

Carbon tetrachloride (CCl4), olive oil and other solvents were purchased from XinXin Glass & Reagent Co. (Xining, China). Bifendate was supplied as a positive control sample by Zhejiang Wanbang Pharmaceutical Co., Ltd (Wenling, China). The references of swertiamarin, gentiopicroside, sweroside, mangiferin, and isoorientin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Chemical analysis with high performance liquid chromatography (HPLC)

The chemical profile of SME-d was recorded by high performance liquid chromatography (Agilent 1100) with diode array detection (DAD). Samples were dissolved by methanol, and the solutions were filtered with 0.45 μm Millipore filters. A reverse phase C18 column (Agilent Eclipse XDB-C18, 250 mm × 4.6 mm, 5 μm) was eluted with the gradient phase (0 min, 18% methanol → 25 min, 55% methanol → 47 min, 80% methanol → 60 min, 100% methanol) at the flow rate of 1 ml/min. The eluate was monitored at the wavelength of 210, 230 and 254 nm, and the column temperature was kept at 25°C. Swertiamarin, gentiopicroside, sweroside, mangiferin, and isoorientin were used as the reference standard.

Hepatoprotective evaluation in rats

KM rats (male and female) 20 to 25 g, were purchased from Laboratory Animal Center, Gansu College of Traditional Chinese Medicine. The animals were maintained at a constant temperature of 23 ± 2°C and fed with tap water and standard laboratory chow (Beijing Ke-Ao-Xie-Li feed. Co., LTD, Beijing, China). Normal group and control group were fed with 20 ml/kg BW distilled water for 8 days orally. Test groups were fed with SME-d at doses of 0.9 and 1.8 g/kg BW for 8 days orally, and positive group was fed with 80 mg/kg BW bifendate for 8 days. Each group contained eight rats. At the 8th day, 10 ml/kg BW CCl4 (0.1%, v/v in olive oil) was administrated by intraperitoneal injection to all groups except normal group. At 22 h after the last dose, all rats were sacrificed. Serum was separated by centrifugating at 3000 rpm for 10 min and used for the measurement of the alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (TBIL), bile acid (TBA) value (Drotman and Lawhorn, 1978). The ALT and AST activities were measured with the ALT and AST Elisa Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The TBIL and TBA contents were determined by the TBIL and TBA Test Kits (Kehua, Shanghai, China).

The effects of SME-d on CYP1A2 and CYP3A4 activities

CYP1A2 and CYP3A4 belonged to the cytochrome P450 superfamily. They are plentiful in liver and responsible for catalyzing the oxidation of organic substances. For drug discovery, researchers need to determine how drug candidate alter P450 activity. In this experiment, all samples were analyzed by P450-GloTM CYP1A2 Screening System (Cat.#V9970) and P450-GloTM CYP3A4 Screening System (Cat.#9910) (Promega, America). CYP1A2 or CYP3A4 enzymes were incubated with their substrates, or and sample for 10 to 30 minutes at 37°C. Reactions were initiated by the addition of a nicotinamide adenine dinucleotide phosphate regenerating solution. P450 activity was stopped and luminescence was initiated by adding luciferin detection reagent. Luminescence was read directly on the FLUOstar OPTIMA microplate reader (BMG LABTECH, Germany) in luminescence mode. 1 μM α-naphthoflavone (sigma, America) were positive control in CYP1A2 screening experiment, and 5 μM Ketoconazole (sigma, America) was in CYP3A4 screening experiment. Firstly, samples were tested in 20 μg/ml dose. If the inhibition of some sample was bigger than 50%, the half maximal inhibitory concentration (IC50) was measured in seven gradients.

Statistical analysis

All statistical analyses were performed by using Microsoft Excel 2000 (Guo, 2000) or the SPSS 10.0 (Mo, 2004) for windows software package. The date were analyzed by Student's t-test to assess the significance of the differences between two means or by one-way analysis of variance (ANOVA) followed by least-significant-difference (LSD) test for more than two means (Milton and Tsokas, 1983). Statistical significance was considered at p < 0.05.

RESULTS

The contents of five hepatoprotective chemical compounds

The contents of sweroside, swertiamarin, mangiferin, gentiopicroside, isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively (Table 1).

The hepatoprotective activity in rat

Compared with normal group, CCl4 induced serum ALT and AST activity in control group significantly (P < 0.01). High and low dose of SME-d and bifendate could inhibit the exaltation of the serum ALT activity induced by CCl4. The inhibition effect of 0.9 g/kg SME-d < 80 mg/kg bifendate < 1.8 g/kg SME-d (Table 2). High and low dose of SME-d could also inhibit the exaltation of the serum AST activity induced by CCl4, but the inhibition of bifendate was not obvious statistically. The effect of 0.9 g/kg SME-d < 1.8 g/kg (Table 2). CCl4 also induced the serum TBIL and TBA content in control group significantly.
Table 1. The contents of five hepatoprotective chemical compounds (mg/g).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sweroside</th>
<th>Swertiamarin</th>
<th>Mangiferin</th>
<th>Gentiopicroside</th>
<th>Isoorientin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SME-d</td>
<td>0.24</td>
<td>3.96</td>
<td>12.30</td>
<td>13.53</td>
<td>16.85</td>
</tr>
</tbody>
</table>

Table 2. The ALT and AST, TBA and TBIL value (mean ± s, n = 6).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>TBIL (µmol/L)</th>
<th>TBA (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>48.67±5.72**</td>
<td>154.33±15.33**</td>
<td>2.65±0.10**</td>
<td>19.84±3.74**</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1952.96±771.75</td>
<td>3130.67±1598.88</td>
<td>6.65±2.71</td>
<td>257.90±95.16</td>
</tr>
<tr>
<td>SME-d</td>
<td>0.9 g/kg</td>
<td>842.33±467.94*</td>
<td>1143.33±493.11*</td>
<td>3.95±0.62*</td>
<td>141.36±13.09*</td>
</tr>
<tr>
<td></td>
<td>1.8 g/kg</td>
<td>605.54±185.45**</td>
<td>1050±253.24*</td>
<td>3.32±0.35*</td>
<td>103.87±24.64**</td>
</tr>
<tr>
<td>Bifendate</td>
<td>80 mg/kg</td>
<td>648.05±177.48**</td>
<td>2222.5±857.93</td>
<td>8.40±2.49</td>
<td>193.38±65.21</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01 vs control.

Table 3. The effects of SME-d on CYP1A2 and CYP3A4 activity.

<table>
<thead>
<tr>
<th>Samples</th>
<th>CYP1A2</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition (20 µg/ml)</td>
<td>IC50 (µg/ml)</td>
</tr>
<tr>
<td>SME-d</td>
<td>99.0±0.267</td>
<td>0.79±0.040</td>
</tr>
<tr>
<td>Bifendate</td>
<td>5.3±6.76</td>
<td>-</td>
</tr>
<tr>
<td>α-naphthoflavone (1 µM)</td>
<td>99.5±0.267</td>
<td>-</td>
</tr>
<tr>
<td>Ketoconazole (5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(P < 0.01). High and low dose of SME-d could inhibit significantly the exaltation of the serum TBIL and TBA content induced by CCl4 (Table 2). The inhibition effect of SME-d showed dose-dependent in 0.9 g kg and 1.8 g/kg (Table 3). Bifendate showed no significant effect on the serum TBIL and TBA content (Table 2).

The effects of SME-d on P450 (CYP1A2 and CYP3A4) activities

SME-d could decrease 99.0 ± 0.267% activity of CYP1A2 in liver cell, and did little influences on CYP3A4. On the contrary, bifendate could inhibit 66.17 ± 2.12% activity of CYP3A4, but affected the activity of CYP1A2 hardly.

DISCUSSION

Some constituents of S. mussotii have been proved to be hepatoprotective (Sun et al., 1991), which contain swertiamarin (Singh, 2008), gentiopicroside (Li et al., 2001), sweroside (Singh, 2008), mangiferin (Liao et al., 2005), isoorientin (Orhan et al., 2003). Measuring these compounds contents in SME-d was a shortcut for understanding its hepatoprotective mechanism. The contents of sweroside, swertiamarin, mangiferin, gentiopicroside, isoorientin were 0.24, 3.96, 12.30, 13.53, 16.85 mg/g in SME-d, respectively. The extract procedure had enriched isoorientin, because the isoorientin content had been measured to be between 2.46 and 7.4 mg/g in S. mussotii (Bao et al., 2006; Li et al., 2008). Moreover, isoorientin could show obvious hepatoprotective function in 15 mg/kg BW (Orhan et al., 2003), and 1.5 mg/kg BW isoorientin should be given in 0.9 g/kg BW of SME-d, so isoorientin should be take parted in the hepatoprotective activity of SME-d. Of course, more works should be done for understanding the hepatoprotective mechanism of SME-d.

Carbon tetrachloride-induced liver injury model was the common model for studying the hepatoprotective medicine. ALT and AST were the sensitive index for the acute liver injury (Fu and Wei, 2005), and TBIL and TBA were the sensitive index for jaundice of liver injury (Zhang et al., 1989). Therefore, the serum ALT, AST, TBA and TBIL were taken as liver injury indicators in rats. CCl4 could induce the ascension of the serum ALT and AST activity, TBA and TBIL content significantly (P < 0.01), which showed the CCl4-induced liver injury model was constructed successfully. SME-d could cut down the exaltation of the serum ALT and AST activity, TBA and TBIL content significantly, which could be deduced that SME-d can perform the hepatoprotective function in rat. Bifendate could decrease the serum ALT activity significantly, and did no obviously influence on the serum AST activity, the serum TBA and TBIL contents, which was similar to...
The difference of SME-d and bifendate should give SME-d more chance to be a new medicine. The CYP3A4 and CYP1A2 belonged to the cytochrome P450 superfamily. They are plentiful in liver and often employed for evaluating the possibility of the interactions in medicines, especially for new medicine. SME-d could inhibit the CYP1A2 activity without effects on CYP3A4, which should give some suggestions for using SME-d correctly. Its difference to bifendate would give hepatitis more choices.

**ABBREVIATIONS**

HPLC, High performance liquid chromatography; ALT, aspartate transaminases; AST, alanine transaminases; TBIL, total bilirubin; TBA, total bile acid; CYP1A2, cytochrome P450 1A2; CYP3A4, cytochrome P450 3A4; SME-d, the alcohol-extraction-water-precipitation fraction of *Swertia mussotii* Franch.; BW, body weight.

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Protective effects of *Launaea procumbens* against oxidative adrenal molecular, hormonal and pathological changes in rats

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The aim of the study was to investigate the protective effects of Launaea procumbens methanolic extract (LM) against CCl\(_4\)-induced molecular, hormonal and pathological abnormalities in rats. Male Sprague Dawley rats were provided by National Institute of Health (NIH) Islamabad and orally fed with 100, 200 mg/kg body weight of LM after 48 h of CCl\(_4\) treatment (3 ml/kg body weight, 30% in olive oil) biweekly for 4 weeks. The results showed that the administration of LM significantly improved the CCl\(_4\)-induced serum level of hormones, argyrophilic nucleolar organizer regions (AgNORs) and DNA damages. Histopathology showed that LM reduced the incidence of adrenal lesions induced by CCl\(_4\) in rats. These results suggest that LM could protect adrenal against the CCl\(_4\)-induced oxidative damage in rats.

**Key words:** Carbon tetrachloride, *Launaea procumbens*, adrenal histopathology.

**INTRODUCTION**

The imbalance between the reactive oxygen species and the ability of biological system to detoxify these reactive intermediate or easily repair the resulting damage causes by them is called oxidative stress. All living organisms maintain a reducing environment within their cells by a system of antioxidant enzymes. This imbalance causes toxic effects through the rapid production of peroxides and free radicals that damage cell and macromolecules including proteins, lipids and nucleic acids. Carbon tetrachloride has molecular formula CCl\(_4\), and its molecular weight is 153.8 g/mol, has been used as solvent in varnishes, resins and as starting material of many industrial organic compounds, and it is estimated that the average daily intake of CCl\(_4\) for a general population is 0.1 μg (Abraham et al., 1999; ATSDR, 2003). Exposure to such toxic chemical through inhalation, ingestion or skin absorption is distributed throughout the body with high concentration in liver, muscles, fat tissue brain, kidney and blood (Ogeturk et al., 2004), and damages various tissues especially liver (Khan and Ahmed, 2009).

Carbon tetrachloride induces reactive oxygen species (ROS) and oxidative DNA damages, with the formation of DNA adducts, genetic mutation, strand breakage and chromosomal alterations. DNA strand breaks are especially important in inducing mutations, such as deletions and translocations in affected cells undergoing replication with error-prone repair or without proper repair. Moreover, extensive DNA strand breaks without prompt repair may cause cell death and compensatory cell regeneration (Khan et al., 2010a, b).

Nuclear morphology can be evaluated histologically using a newly developed silver (argyrophilic) staining method for nucleolar organizer regions (NORs), the so-called AgNOR technique. NORs are composed of...
chromosomal sites endowed with ribosomal DNA (rDNA) and complexes with a set of non-histone proteins characterized by high affinity for silver (Trere et al., 1996; Khan et al., 2010c) used for identification of normal cells from neoplastic cells (Cheah et al., 1996). Medicinal plant play crucial role in improving various pathogenesis (Sahreen et al., 2010; Khan et al., 2009; Khan et al., 2010c). Launaea procumbens is locally used in Pakistan in adrenal dysfunction. Therefore the present study was arranged to evaluate the protective function of L. procumbens versus carbon tetra chloride induced oxidative damages in rats.

MATERIALS AND METHODS

Plant collection and extraction

L. procumbens at maturity was collected from Wah Cantt District Rawalpindi (Pakistan), identified and its ariel parts (leaves, stem, flowers and seeds) were shade dried at room temperature, grinded mechanically and extracted with methanol to get crude methanolic extract. Methanolic extracts were stored at 4°C for in vivo screening.

Animals

Six week old, 30 Sprague Dawley male rats (190 to 200 g) were provided by National Institute of Health Islamabad and were kept in ordinary cages at room temperature of 25 ± 3°C with a 12 h dark/light cycle. They were allowed to standard laboratory feed and water. The study protocol was approved by Ethical Committee of Quaid-i-Azam University Islamabad for laboratory animal feed and care.

Experimental design

To study the protective effects of LM, rats were equally divided into 5 groups (6 rats). Group 1 received only raw water and free access to food materials. Group 2 received olive oil intraperitoneally (Monday and Thursday) and dimethyl sulphoxide (DMSO) intragastric (Wednesday and Saturday) at a dose of 3 ml/kg body weight. Group 3 received CCl₄ 3 ml/kg (30% in olive oil) intraperitoneally (Monday and Thursday). Group 4 and 5 received 100, 200 mg/kg body weight of LM after 48 h of CCl₄ (Wednesday and Saturday), respectively. Experimental period was of four weeks. After 24 h of the last treatment, all the animals were weighted, sacrificed; with their blood collected, weighted and perfuse adrenal in ice-cold saline solution. Half of adrenal tissues were treated with liquid nitrogen for further enzymatic and DNA damage analysis while the other portion was processed for histology.

Assessment of serum markers

Serum hormonal analysis of adrenal gland was carried through kits.

Histopathological studies

For microscopic evaluation adrenal glands were fixed in a fixative (absolute ethanol 60%, formaldehyde 30%, glacial acetic acid 10%) and embedded in paraffin, sectioned at 4 μm and subsequently stained with hematoxylin/eosin. Sections were studied under light microscope (DIALUX 20 EB) at 40 and 100 magnifications. Slides of all the treated groups were studied and photographed.

DNA fragmentations

DNA fragmentation (%) assay was conducted using the procedure of Wu et al. (2005) with some modifications. The adrenal tissue was homogenized in TE solution pH 8.0, centrifuged and separates the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). The pellet and supernatant fractions were assayed for DNA content using a freshly prepared DPA (Diphenylamine) solution for reaction. Optical density was read at 620 nm.

AgNORS analysis

Silver staining technique was used according to Trere et al. (1996) with some modifications. The cells were examined under light microscope at 100× magnification and number of NORs was counted per cell.

DNA ladder assay

DNA was isolated by using the methods of Wu et al. (2005) to estimate DNA damages. 5 μg of rats DNA was loaded in 1.5% agarose gel containing 1.0 μg/ml ethidium bromide including DNA standards (0.5 μg per well).

Statistical analysis

Data were expressed as mean and standard error (SE) and analysis of variance (ANOVA) test was used to analyze the difference among various treatments, with least significance difference (LSD) at 0.05 and 0.01 as a level of significance. SPSS ver. 14.0 (Chicago, IL, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

RESULTS

Effect of L. procumbens on serum level of adrenalin, nor adrenalin and cortisol in rat

The protective effects of L. procumbens against CCl₄ intoxication on serum level of adrenalin, nor adrenalin and cortisol in rat are shown in Table 1. Administration of CCl₄ significantly (P < 0.01) elevated the serum level of adrenalin, nor adrenalin and cortisol as compared to the control group. Serum level of adrenalin, nor adrenalin and cortisol was reversed towards the control group in a dose dependent manner by the treatment of methanolic fraction of L. procumbens.

Effect of AgNORs count and DNA fragmentation

Changes in the effect of L. procumbens against the CCl₄ on AgNORs count and DNA fragmentation in adrenal gland of rat are shown in Table 2. CCl₄ treatment significantly (P < 0.01) AgNORs count and DNA damages
Table 1. Effect *L. procumbens* on serum level of adrenalin, nor adrenalin and cortisol in rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adrenalin (mg/dl)</th>
<th>Nor adrenaline (mg/dl)</th>
<th>Cortisol (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35±3.8**</td>
<td>17.8±0.45**</td>
<td>63±3.65**</td>
</tr>
<tr>
<td>Olive oil+DMSO</td>
<td>36±2.93**</td>
<td>18.86±0.50**</td>
<td>64±3.23**</td>
</tr>
<tr>
<td>3 ml/kg CCl₄</td>
<td>61±6.26**</td>
<td>27.3±0.58**</td>
<td>103±2.12**</td>
</tr>
<tr>
<td>100 mg/kg LM+CCl₄</td>
<td>48±3.11***</td>
<td>21±0.41***</td>
<td>81±3.12***</td>
</tr>
<tr>
<td>200 mg/kg LM+CCl₄</td>
<td>36±2.81**</td>
<td>18±0.18**</td>
<td>66±5.25**</td>
</tr>
</tbody>
</table>

Mean ±SE (n=6 number). *, ** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively. +, ++ indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level, respectively.

Table 2. Effect of various fractions of *L. procumbens* on adrenal AgNORs count and DNA damages.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AgNORs (NORs/cell)</th>
<th>% DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4950±0.0448**</td>
<td>46.67±1.28**</td>
</tr>
<tr>
<td>Olive oil+DMSO</td>
<td>1.6017±0.0744**</td>
<td>47.67±1.09**</td>
</tr>
<tr>
<td>3 ml/kg CCl₄</td>
<td>6.595±0.367**</td>
<td>67.33±0.803**</td>
</tr>
<tr>
<td>100 mg/kg LM+CCl₄</td>
<td>3.835±0.159***</td>
<td>57.50±0.885***</td>
</tr>
<tr>
<td>200 mg/kg LM+CCl₄</td>
<td>2.102±0.215***</td>
<td>48.17±1.30**</td>
</tr>
</tbody>
</table>

Mean ±SE (n=6 number). *, ** indicate significance from the control group at P<0.05 and P<0.01 probability level, respectively. +, ++ indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level, respectively.

as compared to the control group. Treatment of *L. procumbens* significantly (P < 0.01) ameliorated the CCl₄ intoxication and reduced the number of NORs per cell and DNA fragmentation in a dose dependent manner.

**Effect of *L. procumbens* on DNA damages**

Protective effects of different fractions of *L. procumbens* versus CCl₄ induced DNA damages in the adrenal tissues of rats are shown by DNA ladder assay in Figure 1. Extensive DNA breakages in adrenal gland were depicted by the treatment of CCl₄ to rats. Post-administration of *L. procumbens* reduced the DNA damages dose dependently as shown by DNA bands of different groups as compared to CCl₄ group.

**Effect of different fractions of *L. procumbens* on histopathology of adrenal glands in rat**

The microscopic evaluation of adrenal gland sections in control rats showed normal architecture having uniform basophilic nuclei and lack pleomorphism. CCl₄ treatment caused necrosis of adrenal cortex, degradation of modularly cells, accumulation of fatty droplets, damage of structural proteins and the breakage of nuclear membrane. Adrenal medulla showed hypertrophy, hyperplasia and dilation of blood vessels with CCl₄ treatment. Post-administration of *L. procumbens* in the CCl₄ intoxication and reversed necrosis cortex started to attain the normal shape and size and the amount of lipid droplets were also decreased. The nuclear membrane started to repair, medulla size was normal and blood vessels were less dilated than CCl₄ group as shown in Table 3 and Figure 2.

**DISCUSSION**

Our results show that CCl₄ treatment causes significant increase in the secretion of epinephrine, nor epinephrine and cortisol. These changes are markedly restored by treatment of plant extracts. Stern and Brody (1963) reported that the oral administration of 2.5 ml/kg CCl₄ in peanut oil to rats elevated free epinephrine and nor epinephrine levels in plasma and urine, which supports our results. Similarly, results of Rubinstein (1962) are in accordance with our investigations, and reported that intraduodenal administration of carbon tetrachloride to rats for 2 h caused increase in serum epinephrine level. According to the Marnett (2000), the product of lipid peroxidation, MDA react with DNA to form the adduct M1G, the mutagenic pirimedopurinone adduct of deoxyguanosine. According to Shimoda et al. (1994), it is very important to identify risk factors for genomic instability.
Table 3. Effect of *L. procumbens* on histopathology of adrenal glands in rat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adrenal cortex necrosis</th>
<th>Fatty changes</th>
<th>Accumulation of cells</th>
<th>Blood vessels dilation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Olive oil+DMSO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 ml/kg CCl₄</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>100 mg/kg LM+CCl₄</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
<td>-/+</td>
</tr>
<tr>
<td>200 mg/kg LM+CCl₄</td>
<td>-</td>
<td>-</td>
<td>-/+</td>
<td>-</td>
</tr>
</tbody>
</table>

-, normal; -/+, mild; +, medium; +++, severely damaged.

Figure 1. Agrose gel showing DNA damage by CCl₄ and preventive effect of *Launaea procumbens* extracts in different groups. Lanes (from left) DNA marker (M), Control (1-4), CCl₄ (5, 8), 100 mg/kg LM (9, 10) 200 mg/kg LM (11, 12).

Figure 2. Histopathological changes caused by CCl₄ and preventive effect of *Launaea procumbens* extracts in different groups. Slides (from left) Control (A), CCl₄ (B, C), 200 mg/kg LM (D).
which is responsible for the occurrence of genetic alterations for carcinogenesis. The data of the present study revealed that the treatment of CCl₄ causes significant oxidative DNA damage in adrenal gland which are visualized on agarose gel by staining with ethidium bromide. Treatment with *L. procumbens* plant extracts significantly reduces these damages. Similar investigation was reported by Khan et al. (2009) during study of protective effects against carbon tetrachloride induced toxicity in rats.

The microscopic evaluation of adrenal glands showed that CCl₄ treatment caused necrosis of adrenal cortex, degradation of cells, accumulation of fatty droplets, modular hypertrophy, hyperplasia and blood vessels dilation. Similar histopathological changes were reported that carbon tetrachloride causes necrosis to the adrenal cortex after initiation of lipid peroxidation, which requires a CYP-catalysed bioactivation (Rosol et al., 2001).

**Conclusion**

From data it was inferred that protective effects are due to the presence of bioactive compounds in the extract, which might be responsible in modulating the effects of CCl₄-induced toxicity and concomitantly near to normal rats as *L. procumbens* treated groups.

**REFERENCES**


Review

A review on therapeutic potential of *Nigella sativa* (kalonji) seeds

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*Nigella sativa* name as black seed or Kalonji seed belongs to family of rananculacea. It is widely grown in different part of world and is an annual herb cultivated in India and Pakistan, Phytochemically; it contains fixed oil, protein, alkaloids saponin and essential oil. *N. sativa* has been reported to possess potent antioxidant, hepatoprotective, antiparasitic, anticancer, antidiabetic, antimicrobial, antiparasitic, analgesic and anti-inflammatory, anti-nociceptive, anti-ulcer, anti-histaminic etc. The present article reviews on morphology, cultivation, chemical constituent and therapeutic potential as well as clinical aspect and toxicity of *N. sativa* seed.

**Key words:** *Nigella sativa*, morphology, cultivation, chemical constituents, therapeutic potential, clinical aspects and toxicity.

**INTRODUCTION**

Amongst the promising medicinal plant, Kalonji (*Nigella sativa*) a dicotyledonous of rananculacea is an amazing herb with a rich historical and religious background. The seeds of *N. sativa* are the source of the active ingredient of this plant. The actual importance of *N. sativa* to the Muslims came from the holy saying of the Prophet Mohammed “Prayers and peace be upon him” in the black seed is the medicine for every disease except death (Ghaznavi, 1991). It is the same black seed referred by Prophet Mohammed as a panacea (universal healer), that is a remedy for all ailments but cannot prevent ageing or death (Ghaznavi, 1991). Historical use of black seeds has been mentioned in various religious and ethnic books. Black seeds are identified as the curative black cumin in the holy bible; it is also described as the melanthion of Hippocrates and Dioscorides. In the Greco Arab/ Unani-Tibb system of medicine which originate from Hippocrates, his contemporary Galen and Ibn- sina has regarded black seed as a valuable remedy in hepatic and digestive disorder. The famous book of medicine by Ibn-sina *“The cannon of medicine (980-1037)” revealed historical importance of this Black seeds as the seeds “That stimulates the body’s energy and help recovery from fatigue (Ghaznavi, 1991; Chevallier, 1996).

Through thousand of years, until the time being, millions of people in the mediterranean region and Far East countries use the oil of *N. sativa* seeds daily as a natural protective and curative remedy. Historically, it has been recorded that *N. sativa* seeds were prescribed by ancient Egyptian and Greek physicians to treat headache, nasal congestion, toothache and intestinal worm, as well as a diuretic to promote menstruation and milk production (Hajhashemi et al., 2004). In Ayurvedic system of medicine, the seeds are given with butter-milk to obstinate hiccups and are also used in loss of appetite, vomiting, dropsy. They are also used as emmenagogue and galactogogue and as an abortificient in large doses.

In different combinations, the seeds of *N. sativa* have

*Corresponding author. E-mail: stembhurne@gmail.com. Tel: 09922070123.*
been used in obesity and dyspnoea. They have antibilious property and are administered internally in intermittent fever. Constant inhalation of fried seeds releases cold and catarrh. The seeds have also been used in mercury poisoning, sores and leprosy (Ahmad et al., 2004).

**Synonym of black seeds in various languages**

Arabic: Habatut Barakah; Sonez; Habat ut – sauda; Kamune-asvad.
Hindi: Kalonji.
Sanskrit: Krishana – Jiraka.
Persian: Siyadanah (Ahmad et al., 2004; Chevallier, 1996).

**MORPHOLOGY OF THE PLANT**

*N. sativa* is a bushy, self-branching plant of about 50 to 60 cm in height. Leaves are divided into linear segment 2 to 3 cm long; they are apposite in pairs on either side of the stem. Its lower leaves are small, and petiolate and upper leaves are long. The plant has finely divided foliage and pale bluish or white flowers. The flowers grow terminally on its branches. *N. sativa* reproduces with itself and forms a fruit capsule which consist of many white trigonal seeds, once the fruit capsule has matured, it opens up and the seeds contained within are exposed to the air becoming black in colour (black seeds), seeds are triangular in shape, black in colour and possess a severe pungent smell, contains considerable amount of oil (Chevallier, 1996).

**SCIENTIFIC CLASSIFICATION OF THE PLANT**

Kingdom: Plantae.
Subkingdom: Tracheobionta that is, vascular plant.
Supervision: Spermatophyte.
Order: Ranunculales.
Family: Ranunculaceae-Butter cup family.
Genera: *Nigella*.
Species: *sativa*.

**CULTIVATION AND COLLECTION**

The plant is widely grown in different parts of the world and is an annual herb cultivated in India and Pakistan. *N. sativa* is cultivated during winter season in much the same way as wheat. The areas where maize, green gram or black grams are grown can be used after harvesting these crops. Before sowing the seeds, 2 to 3 times ploughing is enough for good crops and weed control.

Heavy soils need more ploughing than light soils. The seeds are sown 30 cm apart. The seeds should not be sown deep because the germination is delayed. About 12 to 15 kg seeds per hectare are sown. Three to five irrigation are required that is, presowing, seeding stage, flowering stage, and fruit formation stage and seeds development stage. Crop matures during April and May. It should be harvested early in the morning. The crop is harvested when the fruit/capsule turn yellowish. The late harvesting may result in shattering the seeds. After harvesting and proper drying it can be threshed by trampling the crop with tractor or proper thresher. After threshing, the seeds should be properly stored in bags or containers (Ahmad et al., 2004).

**Chemical constituents**

In view of its wide range of medicinal uses, the plant has under gone extensive phytochemical studies. *N. sativa* seeds contain 36 to 28% fixed oil, proteins, alkaloid, saponin and 0.4 to 2.5% essential oil. The fixed oil is mainly composed of unsaturated fatty acid that includes arachidonic, eicosadienoic, linoleic and linolenic acid. The saturated fatty acid present in the oil are palmitic, stearic and myristic acid (Hajhashemi et al., 2004).

The essential oil present in the seeds was analyzed by gas chromatography-mass spectrometry (GC–MS). Many components were characterized but the pharmacologically active constituent of volatile oil is thymoquinone (Figure 1a), dithymoquinone, thymol (Figure 1b) and thymohydroquinone (Figure 1c). Dithymoquinone is the dimerised form of Thymoquinone (Ghosheh et al., 1999; Hajhashemi et al., 2004). The crystalline active principle, nigellone is the only constituent of the carbonyl fraction of the oil. The other constituents of the volatile oil of the seed are p-cymene carvacrol, t-anethole, 4-terpineol and longifoline. Four alkaloids have been reported as constituent of *N. Sativa* seeds. Nigelicline (Figure 1d) and nigellidine have an indazole nucleus whereas nigellimine (Figure 1e) and N-oxide of nigellimine are isoquinolines (Atta-ur-Rehman, 1985a, b, 1995). Recently, a triterpene saponin Alfa herein was isolated from the seeds of *N. sativa*. α-heredin (Figure 1f) is known to have antitumor activity (Kumara and Haut, 2001).

The ethanolic extract of the seeds was found to contain three flavonoids namely quercetin and kaempferol 3-glucosyl (1-2) galactosyl (1-2) glucoside and quercetin –3-(6-feruloyl glucosyl) (1-2) galactosyl (1-2) glucoside (Merfort et al., 1997). Other than those triglycoside quercetin 3-glucoside, kaempferol 3-glucoside and rutin were also isolated from the seeds of *N. sativa*.

*N. sativa* seeds contain other ingredient including nutritional components such as carbohydrates, fats vitamins mineral elements and proteins including eight or nine essential amino acid. Fractionation of whole *N. sativa* seeds using sodium dodecyl sulfate polyacrylamide gel...
electrophoresis (SDS-PAGE) shows bands ranging from 94 to 100 KDa molecular mass (Haq et al., 1999). Monosaccharide in the form of glucose rhamnose, xylose and arabinose are also found. The seeds also contain carotene, which is converted by liver to vitamin A, the *N. sativa* seeds are also a source of calcium, irons and potassium (Salem et al., 2000). The summary of the all chemical composition and active principle in *N. sativa* are given in Table 1.

**PHARMACOLOGICAL PROPERTIES OF N. SATIVA SEEDS**

Many studies have been conducted particularly during the last two decades on the effect of *N. sativa* seeds extracts or its active compounds on the various body systems in vivo or in vitro. The following is the selection of some of these studies.

**Antioxidant activity**

Generation of free radicals may be at least partially the basis of many human diseases and conditions. Therefore the antioxidant action of *N. sativa* may explain its claimed usefulness in folk medicine. The essential oil of *N. sativa* was tested for a possible antioxidant activity. The essential oil, thymoquinone and other components like carvacrol, anethole and 4-terpineol demonstrated respectable radical scavenging property. The free radical scavenging effect of thymol, thymoquinone and dithymoquinone were studied on the reactions generating reactive oxygen species such as superoxide anion radical, hydroxyl radical and singlet oxygen using the chemiluminescence and spectrophotometer methods (Kruk et al., 2000). Thymoquinone and fixed oil of *N. sativa* were also reported to inhibit non-enzymatic peroxidation in ox brain phospholipid liposomes (Houghton et al., 1995). The antioxidant effect of thymoquinone (TQ)
Table 1. Chemical composition, including active principles, of *N. Sativa* seed.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sub-group</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed oil (32-40 %) (Gad et al., 1963;</td>
<td>Unsaturated fatty</td>
<td>Arachidonic, eicosadienoic, linoleic, linolenic, oleic and almitoleic acid.</td>
</tr>
<tr>
<td>Babayan et al., 1978; Salama 1973;</td>
<td>acids</td>
<td>Palmitic, stearic and myristic acid.</td>
</tr>
<tr>
<td>Staphylakis and Gegiou 1986)</td>
<td></td>
<td>Fixed oil (32-40 %) (Gad et al., 1963; Babayan et al., 1978; Salama 1973; Staphylakis and Gegiou 1986)</td>
</tr>
<tr>
<td>Volatile oil (0.4-0.45 %) (Enomoto et al., 2001; El-Dakhakhary 1963; Ghosheh et al., 1999)</td>
<td>Saturated fatty acids</td>
<td>Nigellone, thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, α &amp; β-pinene, d-limonene, d-citronellol, p-cymene and 2-(2-methoxypropyl)-5-methyl-1,4-benzenediol6,16-18</td>
</tr>
<tr>
<td>Proteins (Babayan et al., 1978) (16-19.9 %)*</td>
<td>Amino acids</td>
<td>Arginine, glutamic acid, leucine, lysine, methionine, tyrosine, proline and threonine, etc.13</td>
</tr>
<tr>
<td>Alkaloids (Atta-ur-Rehman et al., 1985;</td>
<td></td>
<td>Nigelline, nigellidine, nigellimine-N-oxide</td>
</tr>
<tr>
<td>Atta-ur-Rehman et al., 1995)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coumarins (Atta-ur-Rehman et al., 1985;</td>
<td></td>
<td>6-methoxy-coumarin, 7-hydroxy-coumarin, 7-oxy-coumarin</td>
</tr>
<tr>
<td>Atta-ur-Rehman et al., 1995; El-Zawahry,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1964; Drozed et al., 1973)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponins (Kumara and Haut 2001; Ansari et al., 1988)</td>
<td>Triterpenes, Steroidal</td>
<td>Alpha-Hedrin, Steryl-glucosides, acetyl-steryl-glucoside</td>
</tr>
<tr>
<td>Minerals (1.79-3.74 %) (El-Zawahry, 1997;</td>
<td></td>
<td>Calcium, phosphorous, potassium, sodium and iron</td>
</tr>
<tr>
<td>Babayan et al., 1978)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrates (33.9%) Fiber (5.5 %), Water (6 %) (Haq, et al., 1999; El-Zawahry, 1997)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

and a synthetic structurally related ter-butyl thymoquinone (TBHQ) were examined in vitro. Interestingly, both TQ and TBHQ efficiently inhibited iron dependant microsomal lipid peroxidation in a concentration dependent manner (Badary et al., 2003).

**Hepatoprotective activity**

Hepatotoxicity is associated with alteration in the levels and activities of certain enzymes such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), oxidant scavenger enzymes system including glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT).

The protective action of thymoquinone against the hepatotoxin: terbutyl hydroperoxide has been demonstrated using isolated rat hepatocytes (Daba et al., 1998). In this study, the hepatoprotective activity of thymoquinone (TQ) was compared with that of silybin a known hepatoprotective agent. The mechanism of hepatoprotection of TQ is not certain but may be related to the preservation of intracellular glutathione (GSH), the depletion of which by oxidative stress is known to increase the susceptibility of cells to irreversible injury. It has also been shown that pretreatment of rats with *N. Sativa* oil for 4 weeks was effective in protection against CCl₄ and D-galactosamine induced hepatic damage. No ill effects on liver function were observed when the oil was green orally at a dose of 100 mg/kg/day for 4 weeks. In mice thymoquinone, 8 mg/kg/day for 5 days before and 1 day after CCl₄ treatment was found to protect against the biochemical and histological markers of liver damage (Nagi et al., 1991). Recently, it is also found to show protective effects against ischemia reperfusion injury on liver (Fahrettin et al., 2008).

**Anti nephrotoxic activity**

Administration of seed extract with cysteine, Vitamin E and *Crocus sativa* before administrating the nephrotoxic drug cisplatin was effective in ameliorating the biochemical and physiological indices of nephrotoxicity (El-Dally et al., 1996).
Anticancer activity

Salomi et al. (1992) have shown that the crude methanolic extract of the seeds of this plant exhibited a strong cytotoxic action on Ehrlich ascites carcinoma, Dalton’s ascites lymphoma and sarcoma 180 while exerting minimal cytotoxicity to the normal lymphocytes. In another study, the aqueous and alcoholic extract of N. sativa alone or in combination with H2O2 as an oxidative stressor were found to be effective in \textit{in vitro} inactivating MCF-7 breast cancer cells (Farah and Begum, 2003). The antitumor effect of thymoquinone and β-elemene were investigated both in vivo and \textit{in vitro} in male albino rats on fibrosarcoma induced by 20-methyl cholanthrene and it was found to inhibit tumor incidence and tumor burden significantly. The possible modes of action were discussed as its antioxidant activity and interference with DNA synthesis coupled with enhancement of detoxification process (Badary and Gamal-el-Din, 2001; Zhou et al., 2003; Gali-Muhtasib et al., 2006; Amr, 2009).

A fraction of the ethanolic extract of \textit{N. sativa} seeds was studied in mice against intraperitoneally implanted murine P388 leukemia and subcutaneously implanted Lewis lung carcinoma cells. The life span of treated mice increased by 153% as compared to directly sulphoxide treated control mice. α- Hederin, a triterpene isolated from this fraction, produced significant tumor inhibition rates while the underline mechanism(s) of antitumor activity of hederin remained to be established. Topical application of \textit{N. sativa} and \textit{C. sativa} extracts inhibited two-stage skin carcinogenesis in mice induced by dimethylbenzanthracene and croton oil. The \textit{in vivo} and \textit{in vitro} inhibitory effect of thymoquinone against benzo (a) pyrene induced stomach carcinogeneses are also reported in mice (Salomi et al., 1991). Worthen et al. (1998) have tested \textit{in vitro} a crude gum, a fixed oil and two purified components of the seeds thymoquinone (TQ) and dithymoquinone (DTM) for their cytotoxicity to several parental and multi-drug resistant tumor cell lines.

Antidiabetic activity

Al-Awadi and Gumma (1987) have reported the use of a plant mixture containing \textit{N. sativa}, myrrh, gum, asafoetida and aloe by diabetics in Kuwait. They studied the effect of these drugs for their glucose lowering effect in rats and found it to be effective. Further studies on the plant mixture containing \textit{N. sativa} revealed that the blood glucose lowering effect was due to the inhibition of hepatic gluconeogenesis and the plant extract mixture may prove to be useful therapeutic agent in the treatment of non-insulin dependent diabetes mellitus (Al-Awadi et al., 1991; Mohamed et al., 2009). The volatile oil of \textit{N. sativa} alone also produced a significant hypoglycemic effect on normal and alloxan induced diabetic rabbits without changes in insulin levels (Al-Hader et al., 1993).

In a more recent study, the seed extract when given orally decreased the elevated glucose levels in alloxan induced diabetic rabbits after 2 months of treatment. Another study was designed to investigate the possible insulinotropic properties of \textit{N. sativa} oil in streptozotocin plus nicotinamide induced diabetes mellitus in hamsters. After four weeks of treatment with \textit{N. sativa} oil significant decrease in blood glucose level together with significant increase in serum albumin level were observed (Farah et al., 2002). The study was also confirmed for it protective effects in diabetes for crude extract and n-Hexane extract of \textit{N. sativa} seed (Matira et al., 2008). The clinical study of \textit{N. sativa} on 60 diabetic patients demonstrates significant improvement with reference to total cholesterol, low density lipoprotein cholesterol (LDL- C), and fasting blood glucose indicating effective as an add-on therapy in patients of insulin resistance syndrome (Najmi et al., 2008).

In another study, Nadia and Taha (2009) evaluated the effect of \textit{N. sativa} seed oil and thymoquinone on oxidative stress and neuropathy in Streptozotocin induced diabetic rats. The results indicated to marked increase in norepinephrine and dopamine concentrations and a marked decrease in serotonin concentration compared to...
the control group. These findings were partly reversed by oral administration of either NS oil or TQ.

**Antimicrobial activity**

The antibacterial effect of the phenolic fraction of *N. sativa* oil was first reported by Topozada et al. (1965). The extract and the oil have been reported to have a broad spectrum of activity against a number of microbes. *In vitro* antibacterial effects of the essential oil showed pronounced activity even in 1:1000 dilutions against several organisms that include *Staphylococcus albus, E. coli, Salmonella typhi, Vibrio cholera*. The oil was more effective against gram positive than gram negative organism. El-Kamali et al. (1998) using the plate diffusion method confirmed the report and showed that the essential oil was effective against gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and gram negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) the antibacterial effect was maximal when *Bacillus subtilis* was used. The oil was found to have excellent antifungal activity particularly against *Aspergillus* species. In a study using murine *cytomegalovirus* as a model intreaperitoneal administration of oil substantially decreased the viral load in liver and spleen (Salem et al., 2000).

**Antiparasitic activity**

*N. sativa* oil has been shown to possess anticestodal and antinematodal properties. In a recent study *N. sativa* oil was shown to be effective in reducing the number of *Schistosoma mansoni* worms in the liver and decreased the total number of ova deposited in both the liver and the intestine (Mahmoud et al., 2002; El-Shenawy et al., 2008). *Nigella* has also recently been shown to be effective against other helminths such as *Hymenolepis nana* (Ayaz et al., 2007). It performs this function by augmenting host immunity. Similar protective effects were seen against other worms such as *Trichinella spiralis* and *Aspiculuris* (AbuElEzz, 2005).

**Antimalarial**

Various extracts of *N. sativa* found to show antiplasmodial activity against both *in vivo* and *in vitro* plasmodia infections. It shows 100% inhibition of the parasite growth (*Plasmodium falciparum*) at concentration 50 ug/ml. *N. sativa* shows dose dependant activity against parasite (Abdulelah et al., 2007; El-Hadi et al., 2010).

**Analgesic and Anti-inflammatory activity**

Houghton et al. (1995) reported that crude fixed oil of *N. sativa* and an active principle thymoquinone (TQ) inhibits cyclooxygenase and 5-lipoxygenase pathway of arachi-
nigellone, isolated from *N. sativa*, effectively inhibited the release of histamine from mast cells, possibly through decrease in intracellular calcium and inhibition of protein kinase C (Chakravarti et al., 1993). These effects together with analgesic and anti-inflammatory actions, perhaps can be correlated with the use of *N. sativa* in eczema and asthma, for scorpion and spider stings and for the bites of cat, dog and snake, recommended in the folk medicine (Al-Jishi et al., 2003).

Effect on cardiovascular system

*N. sativa* alone or in combination with honey or garlic are promoted for the treatment of hypertension which drew the attention of El-Tahir et al. (1993) to investigate the action of the volatile oil of *N. sativa* and its active constituent thymoquinone on the arterial blood pressure and heart of anaesthetized rats. Both agents produce a dose dependent decrease in the arterial blood processor and heart rates. These effects were significantly antagonized by atropine, cyproheptadiene and hexamethonium. This suggests that these effects were centrally antagonized mainly via the involvement of 5-hydroxytryptaminergic and muscarinic mechanism. Oral dose of 0.6 ml/kg/day of *N. sativa* extract produced a significant hypotensive effect in spontaneously hypertensive rats. These findings were significantly comparable with the standard anti-hypertensive drug nifedipine (Zaoui et al., 2002). The effect of the drug was concluded to be partially due to its diuretic effect which was comparable to 0.5 mg/kg/day furosemide. In one of study, two-month dietary supplementation with *N. sativa* extract to normal rats has shown a homogenous cardiac hypertrophy and enhanced cardiac contractility at baseline conditions. The hearts of *Nigella*-treated rats developed a moderate but significant hypertrophy that was evident by an increase in the heart weight to body weight ratio. The observed *Nigella*-induced cardiac hypertrophy was associated with an increase in the baseline cardiac inotropic properties (Yar et al., 2008).

Antihyperlipedemic effects

Seeds of *N. sativa* were evaluated in several animals' models for lipid lowering activity in which orally administered extract of seed showed promising activity. It reduces the serum cholesterol and lipoprotein level significantly (Le et al., 2004; El Dakha Khani et al., 2000; Muhammad et al., 2007; Khadiga et al., 2009; Bahram et al., 2009; Ghanya et al., 2010). The study was also conducted on human being by administering the powder of seeds of *N. sativa* before breakfast for two months and was found to reduce the total cholesterol, triglycerides, LDL-cholesterol to a highly significant extent (Inayat et al., 2009; Datau et al., 2010).

Effect on gastro-intestinal tract

In Unani medicine *N. sativa* is used for stomachache and as a digestive, carminative, laxative and anti-jaundice (Chopra et al., 1956). Oral *N. sativa* powder was reported to relieve flatulence. While Nigellone, an active principle of *N. sativa* was found to antagonize histamine induced contractions of guinea pig intestine. In addition, to this a choleretic effect of *N. sativa* oil and its active principles (thymoquinone, thyhydroquinone and dithymoquin- one) reported, respectively (Mahfouz and El-Dakhakhany, 1960). El-Dakhakhani et al. (1965, 2000) investigated the effect of *N. sativa* oil on gastric secretion and ethanol-induced ulcer in rats. Reported to significant increase in mucin content, glutathione level as well as a significant decrease in mucosal histamine content and ulcer formation, with a protection ratio of 53.56%, was found in the *N. sativa* oil pretreated group. More recently, the crude extract of *N. sativa* was shown to cause a dose dependent (0.1 to 3.0 mg/ml) relaxation of spontaneous contractions of rabbit jejunum as well as inhibition of K⁺ - induced contractions in a similar dose range, suggestive of calcium channel blockade (Gilani et al., 2001). Recently, Abdel-Sater (2009) investigated the protective effects of *N. sativa* on hypothyroidism induced development of acute cold stress gastritis in rats.

Effect on respiratory system

El-Tahir et al. (1993) reported that volatile oil of *N. sativa* seeds produce dose dependent increases in the respiratory rate and intratracheal pressure of guinea pig. When the study was conducted only using thymoquinone, in the active principle of volatile, it was found that it only increased the intratracheal pressure without having a significant effect on the respiratory rate, thus the author suggest that volatile oil could be used as potential respiratory stimulant if thymoquinone is removed from the oil. Thus the oil then can be used in Asthma. Gilani et al. (2001) studied the effect of a crude extract of *N. sativa* seed on isolated rabbit jejunum and guinea pig tracheal preparation. The extract was found a dose dependant relaxation of spontaneous contraction in the rabbit jejunum and inhibition of KCl induced contractions. These actions were similar to those produced by verapamil, a Ca⁺⁺ - channel antagonist. The above pharmacological activities of the petroleum ether fraction of the extract were about 10 times higher than those of the crude extract. In an in vitro experiment carried out by Chakravarti et al. (1993) it is suggest that nigellone, a carbonyl polymer of thymoquinone isolated from seeds of *N. sativa* was found to inhibit effectively the histamine release from the mast cells thus showing the basis for its traditional use in Asthma. The results of clinical study of *N. sativa* conducted in children showed to manage the wheeze associated with lower respiratory tract illness.
(Jameel et al., 2009). In another clinical study on forty (40) chemical war victims, Mohammad and Javed (2008) investigated the effect of *N. sativa* on respiratory symptoms. They were recorded symptoms score in three different visits and found significant improvement in all respiratory symptoms score and wheezing in second and third visits compared to first visits.

**Effect on nervous system**

*N. sativa* seeds revealed promising narcotic analgesic activity mediated possibly through opioid receptors (Khanna et al., 1993). The oil from the seeds exhibited central nervous system (CNS) depressant and potential analgesic effect. It was also found to potentiate pentobarbitone induced sleeping time. The study conducted on cultured cortical neurons and influence of neurotransmitters release showed to indicate increased secretion of neurotransmitters. It also modulates amino acid release in cultured neurons. There was increased in GABA activity while secretion of glutamate, aspartate and glycine was found to decrease. All the results represented the sedative and depressive effects of *N. sativa* seed extract (Tarek et al., 2010). Repeated administration of *N. sativa* was also found to decrease the turnover of 5HT and produces anxiolytics activity (Perveen et al., 2009). Thymoquinone is the major constituent of *N. sativa* seeds. In one of the study conducted in mice, thymoquinone reported to show the anticonvulsant activity (Hosseinzadeh et al., 2004; Hosseinzadeh et al., 2005).

**Effect on immune system**

As a natural remedy, people take *N. sativa* seeds or oil is a promoter of good health and for the prophylaxis of common cold and Asthma. In view of that, El-Kadi et al. (1986) investigated the effect of *N. sativa* on immune system and found that the drug has immuno potentiating properties in human T-cells *in vitro*. This was confirmed by Haq et al. (1995) who showed that *N. sativa* seeds activate T-lymphocyte to secrete the interleukin, IL-3 and IL-1B production. In further experiment, they purified the proteins in the whole *N. sativa* seeds and it should be noted that some proteins have suppressive and others have stimulatory properties in lymphocyte culture (Haq et al., 1999).

**Effect on genitourinary system**

The study showed that the volatile oil of *N. sativa* inhibited spontaneous contraction of rats guinea pig uterine smooth muscle induced by oxytocin (Aqel et al., 1996). It was also reported that *N. sativa* crude oil induced uterine contractions both *in vivo* in pregnant rabbits and *in vitro* of non-pregnant rat uteri (El-Naggar and El-Deib, 1992). Similarly, it was found that the hexane extract of *N. sativa* exhibited mild uterotonic activity and prevented pregnancy in rats when given on day 1 to 10 post-coitum (Keshri et al., 1995).

**Effect on reproductive system**

Sixty days study of *N. sativa* seeds shows to increase in the weight of reproductive organs, sperm motility and count in cauda epididymes and testicular ducts. Spermatogenesis was found to increase at primary and secondary spermatocyte. While in fertility, there was increase in number of female pregnant rats (Mukhallad et al., 2009; Al-Sa'aidi et al., 2009).

**Effect on blood**

In view of that the petroleum ether extract of *N. sativa* was studied for its action on blood coagulation and was reported to shorten the whole blood clotting time, plasma clot time and kaolin-cephalin clotting time of male rabbits when compared to control. In addition, a significant shortening of bleeding time in rats was also observed. However, there were no significant effects on the thrombin time or prothrombin time but the partial thromboplastin time was shortened while euglobulin time was prolonged (Ghoneim et al., 1982).

**TOXICOLOGICAL REPORT**

The seed extract and its constituent appear to have a low level of toxicity. The toxicity of fixed oil (10 ml/kg for 12 weeks) of *N. sativa* seeds in mice and rats were investigated through the determination, of LD$_{50}$ values and examination of possible biochemical, hematological and histopathological changes. The low toxicity of *N. sativa* fixed oil was evidenced by high LD$_{50}$ values (11.915 ml/kg), key hepatic enzyme stability and organ integrity values. This suggests a wide margin of safety for therapeutic doses of fixed oil and *N. sativa* seeds. The LD$_{50}$ value of thymoquinone was found to be 2.4 g/kg. Inclusion of thymoquinone in the drinking water of mice at concentration of 0.03% for 90 days resulted in no signs of toxicity except for significant decrease in fasting plasma glucose concentration (Zaoui et al., 2002). In a recent study of diazinon induced organ toxicity, with *N. sativa* seeds extract given orally for three and six weeks, the study observed attenuated extensive changes of hematological and biochemical parameters in diazinon-treated rats. Based upon these results, they suggested *N. sativa* seeds can be considered as a promising therapeutic agent against hematotoxicity, immunotoxicity,
hepatotoxicity, nephrotoxicity and cardiotoxicity induced by diazinon and may be against other chemical pollutants, environmental contaminants and pathogenic factors (Atef and Wafa, 2010). Other studies also demonstrate that treatment with \textit{N. sativa} resulted in significant decrease of haematological disorders induced by aflatoxin (Abdel-Wahhab and Aly, 2005) and cadmium (Demir et al., 2006). No remarkable pathological changes were recorded in bone marrow of animals treated with suspension of \textit{N. sativa} in carbon tetrachloride induced bone marrow toxicity (Abou et al., 2007).

CONCLUSION

\textit{N. sativa} seed and its components are frequently used as a natural remedy for many ailments. A lot of work has been done to evaluate the pharmacological basis of these uses. Most studies confirm its value in folk medicine as analgesic, anti-inflammatory, anti-oxidant, and anti-cancer, anti-microbial, anti-parasitic, antihypertensive and as an immune stimulant. However, controversial results have been reported for its effect on the respiratory system, blood coagulation and uterine motility. More work is needed to determine the pharmacokinetics, biochemical, pharmacodynamic and therapeutics of active components and their interactions with modern drugs and importance to human health with sufficient detail. The ethnomedical approach, if combined with biochemical or physiological methods, would provide useful pharmacological leads.

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Full Length Research Paper

Effects of aluminum toxicity on the growth and antioxidant status in *Jatropha curcas* seedlings

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In the present study, the effects of aluminium (Al) concentrations on growth, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and phenylalanine ammonia-lyase (phenylalanine ammonia-lyase (PAL), EC 4.3.1.5) activities in *Jatropha curcas* L. seedlings were investigated. To carry out such investigations, *J. curcas* embryos were germinated and grown *in vitro* under Al concentrations of 0, 0.5, 1, 2 and 3 mM over a 7-day period. Biomass and the activities of antioxidant defense enzymes, such as SOD, POD, CAT and PAL in *Jatropha curcas* seedlings were observed. Results indicated that with the increasing Al concentrations, the biomass of cotyledons increased initially and then decreased but the biomass of hypocotyls and radicles decreased gradually. The SOD, POD, CAT and PAL activities in the cotyledons, hypocotyls and radicles were mainly increased, but the change trends were different.

Key words: Aluminium (Al) toxicity, *Jatropha curcas*, antioxidative, plant defense system.

INTRODUCTION

Aluminum (Al) is a light metal that makes up 7% of the earth’s crust and is the third element. Al is a major component of the soil and most exists in a fixed status and has no hazard to plants. However, when soils become acidic as a result of natural processes or human activities, the fixed Al turns to soluble forms and the soluble Al could do harm to plants. About 40% of the world’s arable soils are acidic and therefore present Al toxicity hazards (Uexküll and Mutert, 1995). Al toxicity has been considered to be a main limiting factor of crop productivity on acid soil (Foy et al., 1978; Uexküll and Mutert, 1995). The most distinct and earliest symptoms of Al toxicity in plants is the inhibition of root growth, which occurs within hours or even minutes of exposure to Al (Blamey et al., 2004; Dipierro et al., 2005; Kochian et al., 2005; Llugany et al., 1995; Ma, 2007; Ryan et al., 1993). The root meristem has been considered to be the primary site of Al accumulation and toxicity, suggesting that Al interacts with actively dividing and elongating cells (Delhaize and Ryan, 1995), but the mechanism of inhibition of root elongation is not yet well understood (Kopittke et al., 2008; Ryan et al., 1993). In fact, Al can interact with the root cell walls, apoplastic and/or symplastic constituents, disrupt the normal function of plasma membrane and plasma membrane transport system (Ahn et al., 2001; Blamey et al., 2004; Horst et al., 2010; Ishikawa and Wagatsuma, 1998; Jones and Kochian, 1997; Kopittke et al., 2008).

Some reports have shown that the common feature of several metal toxicity symptoms is the enhanced production of reactive oxygen species (ROS) and this results in oxidative stress (Cheng, 2003; Mithöfer et al., 2004; Valko et al., 2005). In order to alleviate oxidative damage, plants have developed comprehensive and integrated antioxidant enzyme and non-enzyme systems. The antioxidant enzyme systems include a series of

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enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), which, together with other enzymes, promote the scavenging of ROS (Alschger et al., 2002; Mittler et al., 2004; Veljovic-Jovanovic et al., 2006). If ROS is in excessive accumulation, it makes the antioxidant enzyme systems and non-enzyme systems of plant disorderly and leads to the oxidation of biomolecules (Boscolo et al., 2003) or even cell death (Cargnelutti et al., 2006).

Al stress, like other metal stress in plants, could lead to oxidative stress (Giannakoula et al., 2010; Schuch et al., 2010; Xu et al., 2011). In most cases, Al was considered to be toxic and had negative effects on plant development (Horst et al., 2010; Sun et al., 2007), but under some conditions, low concentrations can increase growth or produce other desirable effects. Plants that have shown positive growth to Al in nutrient cultures include sugar beet (Keser et al., 1975), tea shrub (Matsumoto et al., 1976), Pinus radiate D. Don (Huang and Bachelard, 1993), Melastoma malabathricum (Watanabe et al., 2005).

Jatropha curcas L. is a drought resistant shrub or tree belonging to the family Euphorbiaceae. Because of the high content of oil in the seeds, Jatropha has been investigated mainly as a potential source of oil and has been recognized as an adequate substitute for fossil oil (Debnath and Bisen, 2008). Before this study, there is little data about Al stress in J. curcas L. (de Macedo et al., 2011). Keeping in view the effects of aluminum toxicity on the growth and antioxidant status in J. curcas seedlings, the present study aimed to investigate the relationship between the concentrations of aluminum (Al) in J. curcas seedlings and the growth, as well as antioxidant enzymes.

MATERIALS AND METHODS

Plant materials and chemicals

Mature J. curcas seeds were collected in August, 2010 from more than 10 individual wild trees in Panzhihua, Sichuan province, China. Seeds were oven dried, selected and stored in a plastic box (Labeled, No. 20100822) and were deposited at 4°C until processing. Other reagents used were of reagent grade or higher.

Embryo germination and seedlings growth

J. curcas seeds were surface sterilized in 70% ethanol for 30 s, and then in 0.1% mercuric chloride for 8 min. Seeds were rinsed with distilled sterile water several times and soaked in sterile water for 24 to 36 h in a culture room. Each embryo was dissected from the seeds on a clean bench. Murashige and Skoog (MS) medium pH was adjusted to 5.8 ± 0.1 prior to autoclaving at 121 ± 2°C for 15 min, with 30 g/L sucrose and 6 g/L agar powder. Culture mediums (25 ml) were dispensed into Wide-neck bottles (100 ml), containing 0, 0.5, 1, 2 and 3 mM Al concentrations. Al was supplemented as AlCl₃. The embryos were placed for germination and growth in in vitro culture for 7 days. The cultures were incubated at 30 ± 2°C under a 12-h photoperiod in cool, white fluorescent light. When the cotyledons of seedlings had developed, cotyledons were washed with double distilled water, blotted and immediately frozen in liquid nitrogen or stored at -80°C for analysis. Three sets of seedlings were analyzed for each Al concentration, with 15 embryos per set.

Protein extraction

Protein extraction of fresh tissues was performed as previously described (Gao et al., 2010). The supernatant was used immediately or frozen and stored at -80°C for assaying of enzyme activity at a later date. Protein was quantified by the Lowry method using bovine serum albumin as standard.

Assay of antioxidant enzymes

POD activity was determined by the Sakharov and Ardila method (Sakharov and Ardila, 1999). One enzyme unit was defined as the amount of enzyme that produced a change of 1 absorbance per min at 470 nm. SOD activity was determined by measuring its ability to inhibit photochemical reduction of NBT (Chen and Pan, 1996). One unit of SOD was defined as the amount of enzyme that caused 50% inhibition of the photo-reduction of NBT under the assay condition. CAT activity was determined by the Montavon method (Montavon et al., 2007). One unit of CAT activity was defined as the amount of enzyme needed to reduce 1 μmol of H₂O₂ per minute. The activities were expressed as unit per gram fresh weight (U/g fw).

Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis for isoenzymes was carried out with 10% acrylamide gel. SOD isoenzyme activity was determined by the Beauchamp and Fridovich method (Beauchamp and Fridovich, 1971). Gels were equilibrated with 50 mM phosphate buffer (pH 7.5) containing 28 μM riboflavin, 28 mM N,N,N,N-tetramethyl ethylenediamine (TEMED) for 30 min, then washed in distilled water for 1 min and resubmerged in the same buffer containing 2.45 mM NBT for 10 to 20 min with gentle agitation in the presence of light. Enzyme bands appeared as colorless bands on a purple background. For the POD isoenzymes activity assay, the gel was soaked in deionized water for 5 min, and then incubated in 0.03% H₂O₂, 0.2% (w/v) benzidine and 0.1% (v/v) acetic acid for 3 to 5 min. When maximum contrast was achieved, the reaction was stopped by rinsing the gel with deionized water (Gao et al., 2009).

Assay of phenylalanine ammonia-lyase (PAL) activity

Enzyme extraction for the PAL activity assay was carried out as previously described (Gao et al., 2010). PAL activity was determined by assaying the reaction L-Phe decomposition product trans-cinnamate, as measured by the increase of absorbance at 290 nm (Hahlbrock and Ragg, 1975). One unit of enzyme activity was defined as the amount of enzyme needed to decrease in absorbance of 0.01 per min. PAL activity were expressed as unit per gram fresh weight (U/g fw).

Statistical analysis

All treatments were arranged in a completely randomized design with three replicates. All data were expressed as means ± standard deviation (SD). Statistical significance was evaluated with a Student’s t-test, and differences were considered significant if P values were less than 0.05.
RESULTS AND DISCUSSION

Effects of Al on plant growth

Figure 1 showed the changes of the fresh weights of cotyledons, hypocotyls and radicles in *J. curcas* seedlings. With the increasing of AlCl₃ concentration up to 1 mM, the fresh weight of cotyledons had a little increase. When AlCl₃ concentration was up to 2 and 3 mM, the fresh weight of cotyledons, compared to the control, was slightly decreased and the changes were not significant. The fresh weight of hypocotyls decreased gradually with increasing Al concentration up to 3 mM and the fresh weight of radicles showed a similar trend, but when Al concentration was higher than 1 mM, the development of radicles was completely suppressed and could be observed, significant morphological aberrations included impaired radicles development, coarser hypocotyls and cotyledons chlorosis (data not shown). Al, at low concentrations, increased the fresh weight of cotyledons. This could not exclude the reason that low Al concentrations might have positive effect to cotyledons, as reported by other researchers (Ma, 2007; Watanabe et al., 2005). With the inhibition of Al toxicity on radicles development, it inhibited the absorption of nutrients and affected the photosynthesis, thereby suppressed the growth of hypocotyls and cotyledons. At the same time, high Al concentrations possibly enhance the ROS production, which led to the oxidative damage to plant cells and blocked the growth. On the base of these results, our findings suggested that high Al concentrations (> 1 mM) can inhibit the normal growth and development of *J. curcas* seedling.

Effects of Al on SOD activities

Al stress, like other abiotic and biotic stress, can induce oxidative stress reactions (Giannakoula et al., 2010). Effects of Al on SOD activity in *J. curcas* seedlings were shown in Figures 2 and 3. Compared to the control, SOD activity in the cotyledons, hypocotyls and radicles was all enhanced by Al stress. SOD activity in the hypocotyls increased significantly with increasing Al concentrations, and the maximal levels increased by 98.1% when Al concentration was 3 mM. In the cotyledons and radicles, the SOD activity increased by 55.1% and 28.2% at Al concentrations of 2 and 1 mM compared to the control, respectively. Many research have also indicated that Al could increase SOD activity in plants (Schuch et al., 2010; Du et al., 2010; Li et al., 2011) and this may be due to Al inducing the cell to initiate SOD synthesis to remove the superoxide radicals (Giannakoula et al., 2010). However, when the amount of free radicals exceeds cell’s capacity, enzymatic activities start decreasing and if unchecked could ultimately lead to DNA damage (Meriga et al., 2004). The significant increase of SOD activity in our study may be induced by the increased production of ROS and can be a defensive mechanism developed by *J. curcas* seedling against stress. The pattern of SOD isoforms was analyzed by native PAGE, and activity staining revealed that at least four SOD isoenzyme bands in the cotyledons, hypocotyls and radicles were detected, respectively (Figure 3A to C).

The staining intensities of isoenzyme I and II in the cotyledons, hypocotyls and radicles were induced with increasing Al concentrations, but isoenzyme III and IV had virtually no change. The different expression of SOD genes may due to the subcellular location of the enzyme, the upstream sequences in the genomic sequences and
the environmental stress (Mittler et al., 2004). The staining intensities of these isoenzymes showed a similar change compared to the changes of SOD activity assayed in solutions (Figures 2 and 3). With the increase of Al concentrations, the SOD activities were induced and this may promote the tolerance of *J. curcas* seedlings against Al stress.

**Effects of Al on POD activities**

Effects of Al on POD activity in the cotyledons, hypocotyls and radicles were shown in Figure 4. Al stress significantly affected the POD activity in the cotyledons with an increase of 270% when Al concentration was up to 3 mM. At the same time, POD activity in the hypocotyls and radicles was also induced, with the maximum increases of 33.4 and 37% compared to the control when Al concentration was 3 and 1 mM, respectively. As an important enzyme in plant defense system, POD can play an important role when plant is in adverse condition, and multiple POD isoforms have been found in many plant species (Passardi et al., 2005). The expression pattern of *J. curcas* seedling was shown in Figure 5. On the activity gels, at least six bands in the cotyledons, hypocotyls and radicles were observed. POD isoenzyme (II and III) in the...
cotyledons showed an increase in the staining intensities with the increasing of Al concentration. In the hypocotyls and radicles, the main increase in the staining intensities was isoenzyme IV and III, respectively.

According to Figure 4 and 5, the changes of total staining intensities on activity gels and POD activity assayed in solutions were similar. In Al stress response, POD isozymes might consist in scavenging the toxic lipid hydroperoxides generated by the peroxidation of membrane lipids and they could participate in lignin biosynthesis to build up the physical barrier against toxic metals entering the cell (Ezaki et al., 1996; Hegedüs et al., 2001). Our findings suggested that POD, together with SOD and CAT, can increase their activities when J. curcas seedlings were exposed to Al stress, and they could scavenge ROS and reduce the damage caused by Al stress.

Effects of Al on CAT activities

CATs and PODs are the two major systems for the enzymic removal of hydrogen peroxide (H$_2$O$_2$) in plants, and CATs have mainly been associated with the removal of H$_2$O$_2$ in peroxisomes (Willekens et al., 1995). The changes of CAT activities in J. curcas seedlings exposed to Al stress were shown in Figure 6. Compared to control, CAT activities in hypocotyls and radicles were all increased, with the maximum increases of 46.4 and 10.7% when Al concentration was 2 and 1 mM, respectively. In cotyledons, CAT activities were increased first and then decreased with the increasing Al concentration. When the Al concentration was 0.5 mM, the CAT activity in cotyledons was the highest with increase by 58% but when concentration increased to 3 mM, the activity was lower than control. The CAT activity in the hypocotyls and radicles was very low; this might indicate that H$_2$O$_2$ degradation occurred due to POD rather than CAT. Similar results have already been observed in maize exposed to Al stress (Boscolo et al., 2003).

According to Figures 4 and 6, at low Al concentration, the removal of H$_2$O$_2$ in the cotyledons was mainly due to CAT rather than POD and at high Al concentration, this situation was just opposite. The decrease in CAT activity at highly Al stressed seedlings might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under such conditions (Sharma and Dubey, 2007). Our findings suggested, at least here, that CAT appeared not to be an effective ROS-scavenger exposed to Al toxicity.

Effects of Al on PAL activities

PAL, a key enzyme involved in the metabolism of phenolics and lignification of cell walls, was mainly involved in defense mechanisms (Kovacik and Backor, 2007). Effect of Al on PAL activity in the cotyledons, hypocotyls and radicles are shown in Figure 7. Compared to the control, the PAL activities were all increased but the change trends were different. In the cotyledons and radicles, PAL activities were increased first and then decreased with the increasing Al concentration, and when exposed to 0.5 mM Al, the activity was the highest which increased by 37.2 and 23.4%, respectively. In the hypocotyls, the maximum activity was observed at 3 mM Al and the increase was 149.9%. Similar changes were also observed in the previous studies (Kovacik and
Figure 6. Effects of Al on catalase (CAT) activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (*n* = 3).

Figure 7. Effects of Al on phenylalanine ammonia-lyase (PAL) activity in the cotyledons, hypocotyls and radicles of *J. curcas* seedlings grown in MS medium containing 0.5, 1, 2 and 3 mM Al. Values are the means ± SD (*n* = 3).

Some researchers indicated that PAL enhancement in the environmental stressed conditions is due to H$_2$O$_2$ generation which occurs as primary reaction in response to stress (Dorey et al., 1999). So, our results suggested that increased PAL activities may be related to *J. curcas* seedlings response to Al stress.

**Conclusion**

The changes of SOD, POD, CAT and PAL activity were studied when *J. curcas* seedlings were exposed to different Al concentration and the expression patterns of SOD and POD were also shown based on *in vitro* embryo germination and culture. The results in this study showed...
that the increases of SOD, POD, CAT and PAL activity might be an important part of \textit{J. curcas} seedlings resistance mechanisms to Al stress and the synergistic effects might help to reduce the accumulation of ROS and the oxidative damage. This research might provide some evidences for further study into the response mechanisms of \textit{J. curcas} to Al stress.

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**REFERENCES**


Full Length Research Paper

In vitro antibacterial activity and phytochemical analysis of some medicinal plants

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Essential oils of medicinal plants have been used traditionally against pathogenic bacteria that caused infectious disease in human and microbial spoilage of food and have been used safely in herbal medicine as antibacterial compounds. In the present study, the antibacterial activities of the oils were evaluated against human and animals pathogenic bacteria. In this assay, the selective plants reported ethnobotanical uses traditionally and also were referenced in some herbal medicine text. The essential oil of Stachys pubescens, Mentha piperita, Clinopodium vulgare and Satureja hortensis were prepared by hydrodistillation and were analyzed by gas chromatography/mass spectrometry (GC/MS). The number 23, 22, 21 and 21 components were identified in S. pubescens, S. hortensis, M. piperita and C. vulgare, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of oils were determined with broth microdilution and agar diffusion method on bacterial strains. Results from the antibacterial testing indicated that S. pubescens, M. piperita and C. vulgare essential oils showed high activities and inhibited the growth of all the selected bacteria. While the essential oil of S. hortensis displayed the moderate potential activity. Our finding supported the notion that plant essential oils composition or total extract may have a role as pharmaceuticals and preservatives effects as safely and effective drugs with low resistance against microorganisms. Therefore, these essential oils could be used for management of these pathogens as a potential source of sustainable eco-friendly botanical bactericides.

Key words: Antibacterial, Stachys pubescens, Mentha piperita, Clinopodium vulgare, Satureja hortensis, gas chromatography/mass spectrometry (GC/MS).

INTRODUCTION

Herbal medicine has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources as a good choice, because these natural resources have ordinarily fewer side effects (Zargari, 1996). The medicinal plants have been proved effective in the treatment of infectious diseases and simultaneously decrease many of the side effects (Iwu et al., 1999). Also, they are costless and effective against a broad spectrum of antibiotic resistant microorganisms and they have very potent natural biologically active agents (Nychas et al., 2003). In many parts of the world, the extracts and essential oil of medicinal plants with active biological compounds are used for their antimicrobial and antiviral properties (Hassawi and Kharma, 2006) that have been used in folk medicine. The increasing occurrence of
Antimicrobial resistance represents a worldwide major concern for both human and veterinary medicine (Lorian, 1996). For this reason, there is a growing interest in the antimicrobial screening of extracts and essential oils from plants in order to discover new antimicrobial agents. Nowadays, about 25% of the drugs prescribed worldwide come from plants and 252 of them are considered as basic and essential by the World Health Organization (WHO). The WHO considers phytotherapy in its health programs and suggests basic procedures for the validation of drugs in developing countries. Infectious diseases are the second leading cause of death worldwide (Fazly-Bazzaz et al., 2005). From the time of the ancient Iranian, the plants were considered to protect against diseases. Iran has a very honorable past in traditional medicine, which goes back to the time of Babylonian, Assyrian civilization. One of the most significant ancient heritages is sophisticated experience of people who have tried over millennia to find useful plants for health improvement, with each generation adding its own experience to this tradition (Naghibi et al., 2005). Based on literature search, 18% of the plant species are used for medicinal purposes in Iran. Treatment of infections continues to be a problem in modern time, because of side effects of some drugs and growing resistance to antimicrobial agents. To investigate for novel, safer and more potent antimicrobials is a pressing need. Herbal medicines have received much attention as a source of new antibacterial with low side effect and significant activity (Fazly-Bazzaz et al., 2005).

In the present study, the biological activities of four plants: Stachys pubescens, Mentha piperita, Satureja hortensis and Clinopodium vulgare were evaluated. Nowadays, there is a considerable research interest towards the compositional analysis of essential oil and extract. It has been reported that essential oil yield and their components in plants is related to genetic (Mohammed and Al-Bayati, 2009), climate, elevation, topography (Pourohit and Vyas, 2004; Rahimmalek et al., 2009a) and genotype (G), growing conditions (E) and their interaction (G × E) (Basu et al., 2009; Shafie et al., 2009). Previous studies have shown that these selected plant species have potential medicinal activity (Iscan et al., 2002; Mimica-Dukić et al., 2003; Saeed and Tariq, 2005; Andoğan et al., 2002; Mathur et al., 2011; Hammer et al., 1999; Adam et al., 1998; Sahin et al., 2003; Gulluce et al., 2003; Razzaghi-Abyaneh et al., 2008; Adinguzel et al., 2007; Boyraz and Özcan, 2006; Dikbas et al., 2009; Azaz et al., 2005; Chorianopoulos et al., 2004; Mihajilov-Krstev et al., 2010; Mihajilov-Krstev et al., 2009; Karami-Osboo et al., 2010). The antibacterial activity of essential oil of S. pubescens and C. vulgare has never been evaluated and was carried out for the first time in this study. The widespread use of antibiotics both inside and outside medicine is playing a significant role in the emergence of bacterial resistant (Goossens et al., 2005). Although, there were low levels of preexisting anti-biotic resistant bacteria before the widespread use of antibiotics; evolutionary pressure from their use has played a role in the development of multidrug resistance varieties and the spread of resistance between bacterial species (Hawkey and Jones, 2009). Biological cost or metabolic price is a measure of the increased energy metabolism required to achieve a function. Drug resistance has a high metabolic price (Steven and Timothy, 2010) in pathogens for which this concept is relevant (Wichelhaus et al., 2002). Although several strategies have been proposed to overcome and control this situation. However, a clear solution has not yet been elucidating due to the antibiotic resistance, consequences, and side effects of antimicrobial drugs. Many plants are used in Iran in the form of oils and crude extracts, infusion or plaster to treat common infections without any scientific evidence of efficacy. Pharmacological studies carried out on essential oils of some aromatic plants’ species that were obtained in central regions of Iran, have shown antimicrobial activity which is coherent with the use of these plants in folk medicine. It is interesting to determine whether their traditional uses are supported by actual pharmacological effects or merely based on folklore. In the present study, three medicinal plants were selected which are widely used in the folk medicine in our region. All the plants have been used in the treatment of infectious diseases with different geographical area (Rechinger, 1982a, b; Chevallier, 1996). The aim of this study was to evaluate the antibacterial potential of the essential oils derived from S. pubescens, M. piperita, S. hortensis and C. vulgare that grows in the wild in the central part of Iran against standard strains. The selected strains: Staphylococcus aureus (PTCC:1431), Listeria monocytogenes (PTCC:1163), Streptococcus pneumoniae (PTCC:1240), Pseudomonas aeruginosa (PTCC:1430), Klebsiella pneumoniae (PTCC:1053), Escherichia coli (PTCC:1329) and Salmonella typhi (PTCC:1609) were purchased from Iranian Research Organization for Science and Technology (IROST). The antimicrobial potential was performed by disc diffusion (DD) and broth microdilution method (BMD) to determine the minimum inhibitory concentration (MICs) and maximum bactericidal concentration (MBCs).

MATERIALS AND METHODS

Collection of plant and essential oil extraction

The plants were collected from their wild habitat in Semnan city in the central part of Iran between April and June, 2011 which are shown as geographical and environmental conditions in Table 1. Plants were identified by experts of the University of Applied Science and Technology (UAST) Education Center in Semnan branch. The leaves of S. pubescens, M. piperita, S. hortensis and C. vulgare in full flowering stage were collected to determine antibacterial activity. A voucher specimen for each plant has been deposited in the herbarium of Medicinal Plants Research, UAST.
Table 1. Geographical and environmental conditions.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Plant</th>
<th>Region</th>
<th>Altitude (m asl)</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Mentha piperita</td>
<td>Garmsar, North Eeyvanakey</td>
<td>2100</td>
<td>35.432670</td>
<td>53.256050</td>
</tr>
<tr>
<td>2</td>
<td>Satureja hortensis</td>
<td>Shahrood Mayamey Bekran</td>
<td>2150</td>
<td>36.43520</td>
<td>54.376050</td>
</tr>
<tr>
<td>3</td>
<td>Clinopodium vulgare</td>
<td>Semnan Fullad mahaleh</td>
<td>2650</td>
<td>35.78527</td>
<td>53.32405</td>
</tr>
<tr>
<td>4</td>
<td>Stachys pubescens</td>
<td>Shahrood, Semnan</td>
<td>2315</td>
<td>36.32415</td>
<td>54.35316</td>
</tr>
</tbody>
</table>

Air-drying of plant material was performed in a shady place at room temperature for 4 days. Ground and dried leaves of plants (100 g) were subjected to hydro-distillation for 3 h, using a Clevenger-type apparatus. The distilled oils were dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4°C until use.

Gas chromatography/mass spectrometry (GC/MS) analysis

The essential oils were analyzed on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. A fused silica capillary column DB-5 (30 μm, 0.25 mm i.d, film thickness 0.25 μm) and a flame ionization detector (FID) was used for the separation. Helium was used as a carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed at 60°C (4 min), and then rising to 300°C at 4°C min⁻¹. The injector and detector temperature were kept at 250 and 300°C, respectively. The mass spectrometer was operated in electron-impact ionization (EI) mode with 70 eV energy with MS transfer line at temperature of 300°C was used. Ion source and interface temperatures were 200 and 250°C, respectively. The split ratio was 1:50. The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250°C. The column was programmed as follows: 60°C for 2 min and then increased by 3°C min⁻¹ up to 300°C. Volume of injected samples was 0.5 μl. Identification of components was based on the comparison of retention times (RT) and the computer mass spectra libraries using Wiley 275 GC/MS Library (Wiley, New York), those found in the literature (Adams, 2001; McLafferty, 1993) and the mass spectrometry data bank (NIST). The percentage composition of the essential oil was computed by the normalization method from the GC peak areas measurements (Table 4).

Microorganisms, inoculums and antibacterial assay

Bacterial strain

In the present study, a total of 7 standard isolates were obtained from IROST in 2011. Bacterial strains used in this study were four Gram-negative bacteria: P. aeruginosa (PTCC: 1430), K. pneumoniae (PTCC: 1053), E. coli (PTCC: 1329), S. typhi (PTCC: 1609) and three Gram-positive bacteria: S. aureus (PTCC: 1431), L. monocytogenes (PTCC: 1163), S. pneumoniae (PTCC: 1240), that were grown in Mueller-Hinton (MH) agar (Oxoid) and incubated for 24 h at 37°C. Cultures were used for making bacterial suspensions, and turbidity was adjusted to 0.5 McFarland and confirmed using a spectrophotometer (UV-VIS 1650, Shimatzu, Japan).

Preparation of inoculums

Bacterial strains were prepared by suspending one isolated colony from MH agar plates in 5 ml of MH broth and overnight broth cultures. The suspensions were adjusted in 0.5 McFarland standard turbidity to obtain final inoculums of 5 x 10⁵ to 5 x 10⁶ CFU/ml after 24 h of growth at 37°C and confirmed using a spectrophotometer. The essential oils were dissolved in dimethyl sulfoxide (DMSO, 25 mg/ml) and diluted to MH broth for antibacterial tested. All strains were tested by BMD and disk diffusion (DD) techniques according to the National Committee for Clinical Laboratory Standards (NCCLS, 2003a, b).

Serial dilution method

MICs and MBCs of essential oils were determined by using BMD method as described by NCCLS (2003a) in flat-bottomed 96-well clear plastic tissue-cultured plates (Greiner, 650161). The MIC was assayed using two-fold BMD method in MH Broth in 96-well plates. Plates contained two-fold dilutions of antibacterial agents at the concentration ranges: 0.5 to 64 μg/ml (25%, v/v). These dilutions were used to dispense 100 μl into each of the sterile 96-wells and an equal volume of bacterial inoculums was added to each well on the microtiter plate. After incubation for 24 h at 37°C, the micro-dilution trays were checked with unaided eyes to detect the growth inhibition of the bacteria, and then the MICs were determined with spectrophotometer. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The final concentration of DMSO in the assays did not interfere with the bacterial proliferation which is used as a control. Negative controls were prepared with non-inoculated medium with oils, and one non-inoculated well, without antimicrobial agents, was also included to ensure medium sterility. The commercial antimicrobials Ciprofloxacin (Sigma) and Gentamicin (Merk) were included as positive controls. One inoculated well was included to allow control of the broth suitability for organism growth. To determine the MBCs, the suspensions (20 μl) were taken from each well without visible growth and inoculated in MH agar for 24 h at 37°C. The MBC was defined as the lowest concentration of the essential oil at which incubated micro-organisms are completely killed. Tests were performed in triplicate for each test concentration (P > 0.05).

Disc diffusion method

Agar diffusion method was carried out for the assessment of the essences antibacterial activity as recommended by NCCLS (2003b). The potential activity of oils were confirmed by the inhibitory effect on bacterial growth as reflected by the inhibition zone (IZ) compared to known standard antibiotics. Essential oils were diluted in DMSO to different concentrations (0.5, 1.2.4.8.16, 32 and 64 μg/ml). 50 μl of standardized inoculums according to 0.5 McFarland turbidity standard solutions (10⁵ to 10⁶ CFU/ml) of the selected strains were spread onto the surface of Mueller Hinton (MH) agar and kept for 2 h at 4°C for absorption. Sterilized paper discs (Whatman, 6 mm diameter) containing approximately 20 μl of the essential oils were impregnated with different amount of essential oils. The prepared discs of the oils and standard antibiotics were placed on the surface of MH agar media. The inoculated plates were incubated at 37°C for 24 h and the resulting
zyme of inhibition (diameter) was measured in millimeters by comparing the different concentrations of oils and the standard antibiotics. The MIC was defined as the lowest concentration, resulting in a clear zone of growth inhibition around the disc after incubation period. Gentamicine (Merk) and Ciprofloxacin (Sigma) discs were applied over the test plates as a positive control. Negative controls were prepared using the solvent to dissolve the essential oil solution. All experiments were performed in triplicate.

Statistical analysis

Comparison of data was performed using the one way analysis of variance (ANOVA) or the unpaired Student’s t-test and is presented as mean ± standard deviation. Comparison of MIC and MBC values, tests were made in triplicate for quantification. Values of p < 0.05 were considered significant.

RESULTS

All essential oils showed effective antibacterial activities on the selected pathogenic bacteria. Antibacterial activities of essential oils were investigated by broth microdilution and the disc diffusion method. The MICs and MBCs and diameter of inhibition zone (DD) of the selected oils on the bacteria are shown in Tables 2 and 3. The results showed that essential oil of the plants were active against all the pathogenic bacteria species with different degree in the following range of concentrations: essential oil of S. pubescens and M. piperita had the best antibacterial effect and its MIC value was between 0.5 and 8 μg/ml. C. vulgar had the second degree with MIC values between 0.5 and 16 μg/ml. Otherwise, S. hortensis had a lowest antibacterial effect comparison to the earlier essences and its MIC value was 1 to 32 μg/ml. Ciprofloxacin and Gentamicin used as positive control as well as DMSO as a negative control which did not show any inhibition against the pathogens bacteria. MIC range of standard antibiotics “Ciprofloxacin and Gentamycin” were 0.5 to 1 μg/ml and 0.5 to 2 μg/ml, respectively. Even at low concentrations, the plant’s species showed antibacterial activity more or nearly equal to the commercial bactericidal agents. All of the oils had the best inhibitory activities against S. Pneumonia, S. aureus and P. aeruginosa. The weakest activity was observed against L. monocyto genes and K. pneumoniae with the highest MIC and MBC, and K. pneumoniae was resistance against S. hortensis. The results of the chemical analyses using GC/MS of the essential oils were listed in Table 4. Number of indentified constituents

Table 2. MIC and MBC (μg/ml) values for different essential oils of plants.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gentamicin</th>
<th>Ciprofloxacin</th>
<th>M. piperita</th>
<th>C. vulgar</th>
<th>S. hortensis</th>
<th>S. pubescens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MBC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MBC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MBC&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>S.p</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S.a</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>P.a</td>
<td>1</td>
<td>1</td>
<td>0.125</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>E.c</td>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>S.t</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>L.m</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>K.p</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>16</td>
</tr>
</tbody>
</table>

MIC=Minimum inhibitory concentration; MBC= minimum bactericidal concentration; "-" No growth inhibition. E.c=Escherichia coli, P.a= Pseudomonas aeruginosa, S.a=Staphylococcus aureus, S.t=Salmonella typhi, S.p=Streptococcus pneumonia, K.p=Klebsiella pneumoniae, L.m=Listeria monocytogenes.

Table 3. Antibacterial activity screening of antibacterial agents by zone of inhibition (mm diameter) in disc diffusion method.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>D.D of NC</th>
<th>D.D of PC</th>
<th>M. piperita</th>
<th>C. vulgar</th>
<th>S. hortensis</th>
<th>S. pubescens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>C</td>
<td>D.D&lt;sub&gt;T&lt;/sub&gt;</td>
<td>D.D&lt;sub&gt;T&lt;/sub&gt;</td>
<td>D.D&lt;sub&gt;T&lt;/sub&gt;</td>
<td>D.D&lt;sub&gt;T&lt;/sub&gt;</td>
</tr>
<tr>
<td>*S. pneumonia</td>
<td>-</td>
<td>18</td>
<td>25</td>
<td>27</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>**S. aureus</td>
<td>-</td>
<td>24</td>
<td>28</td>
<td>25</td>
<td>27</td>
<td>18</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>-</td>
<td>19</td>
<td>22</td>
<td>23</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>E. coli</td>
<td>-</td>
<td>14</td>
<td>16</td>
<td>17</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>***S. typhi</td>
<td>-</td>
<td>17</td>
<td>21</td>
<td>16</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>L. monocyto genes</td>
<td>-</td>
<td>16</td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>-</td>
<td>15</td>
<td>18</td>
<td>8</td>
<td>7</td>
<td>-</td>
</tr>
</tbody>
</table>

D.D= Diameter of inhibition zone (mm) including of disc diameter of 6mm. T=tested at a concentration of 20 μg/disc. NC=Negative Control. PC=Positive Control (G=Gentamicin, C=Ciprofloxacin, ) -" No growth inhibition. *S=Streptococcus, **S=Staphylococcus, P=Pseudomonas, E=Escherichia, ***S=Salmonella, L=Listeria, K=Klebsiella.
The best antibacterial activities were observed for M. piperita, S. pubescens, S. hortensis and C. vulgaris, respectively. Also, analysis of data with creditability shows that the main components of S. pubescens were: Germacrene (22.4%), δ-Cadinene (19.7%), 2,6-Octadien (11.5%), Linalool (9.7%); M. piperita were: Menthofuran (25.70%), Menthol (23.17%), Menthone (18.68%) and Camphane (7.40%), C. vulgaris were: Germacrene D (18.12), β-pinene (16.04), Sabinene (10.19) and 1,8-cineole (6.76); and S. hortensis were Thymol (48.58%), α-Cymene (16.91%), Carvacrol (5.19%), trans-Caryophyllene (3.72%) and γ-Terpinene (3.56%).

**DISCUSSION**

This study was attempted to purify the selected plant's oils that were native in our region in order to identify their essential oils as antibacterial properties. In addition, components of plants were determined and the result was compared with other studies. This is due to several reasons, namely, conventional medicine can have side effects, high cost, abusive or incorrect usage of synthetic drugs result in complications, and the large percentage of the world's population do not have access to conventional pharmacological treatment. The best antibacterial activities were seen in *M. piperita, S. pubescens* and *C. vulgaris*.
while *S. hortensis* displayed a moderate response against bacterial species. In comparison to the standard drugs, these data showed *M. piperita* and *S. pubescens* had the highest activity; *C. vulgare* had the lower activity but with the lowest different, while the different properties of *S. hortensis* was more. The results confirmed the antibacterial potency of these plants. In other studies concerning the antimicrobial activity of these plants, inhibition effects of *M. piperita* on some microorganisms such as *S. paratyphi*, *Proteus mirabilis*, *Proteus vulgaris*, *Streptococcus mutans*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Shigella dysenteriae* and *Yersinia enterocolitica* growth was studied and this plant showed the highest antimicrobial activity (Iscan et al., 2002; Mimica-Dukić et al., 2003; Saeed and Tariq, 2005; Mathur et al., 2011; Andogan et al., 2002). In previous studies, *S. hortensis* showed antimicrobial activity against some of the standard and clinical microorganisms (Iscan et al., 2002; Sahin et al., 2003; Gulluce et al., 2003; Adinguzel et al., 2007; Boyraz and Özcan, 2006; Azaz et al., 2005; Chorianopoulos et al., 2004; Mihajlov-Krstev et al., 2010; Mihajlov-Krstev et al., 2009; Karami-Osboo et al., 2010; Baser et al., 2004).Other study concerning the extract of *C. vulgare*, inhibited the growth of some bacterial species with different degrees (Opalachnova and Obreshkova, 1999). Our result with other studies confirmed that a variety of bacterial species are affected by essential oil of selected plants, especially about the essential oils of *S. pubescens* and *C. vulgare* evaluated for the first time. The aforementioned finding supports their traditional usage of these oils as antibiotic and antiseptic (Riley, 2005). Briefly, the results of this study showed that the essential oils of these plants have a very broad spectrum of antibacterial activities with notable MICs and MBCs, which are near or lower than dose synthetic drugs. These plants could safely be used as organic preservatives to replace synthetic antibiotics in the prevention and cure of some human and animal infectious disease as well as food industrial preservatives. However, it is necessary to determine the toxicity of the active constituents, their side effects and pharmaco-kinetic properties. In comparison to the other studies, the composition of the plants showed little difference. Similar to our result, previous studies on *M. piperita* showed that the main components of the oils and extracts were menthol, menthofuran, menthone and menthyl acetate (Iscan et al., 2002; Maffei et al., 1999; Sokovic et al., 2009; Rohloff, 1999). There are three numbers of these components in our main components, but in the other study in Iran, the main components were very different: α-terpinene, isomenthone, *trans*-carveol, pipertittinone oxide (Rasooli et al., 2006). Menthone and menthol has been reported to be responsible for the antimicrobial activity of *M. piperita* (Gupta and Saxena, 2010; Bassolé et al., 2010; Kizil et al., 2010). But, the antibacterial activity of menthofuran has not been deter-
inhibited both Gram positive and Gram negative bacteria (Dikbas et al., 2006; Hoferl et al., 2009). It was suggested that these differences in components could be due to the variety of the ecotype system reported by other scientists and references (Asbaghian et al., 2011). Since the essential oils are complex mixtures of several compounds, it is difficult to attribute their biological activity to a particular constituent. Usually, major compounds are the ones responsible for the antimicrobial activity of the essential oils. However, some studies showed that minor components may have a crucial role in the biological activity of the oils (Koroch et al., 2007). Further studies are needed to determine the antibacterial activities of the bioactive compounds responsible for the observed potential value. Natural plant-derived bactericidal may be a less alternative active compounds. In an attention to, in the present study, most isolates showed a different concentration of essential oils between bacteriostatic and bactericidal values. Suggesting that the essential oils of the selected plants could be a possible source to obtain new and effective herbal medicines to treat infections caused by multi-drug resistant strains of microorganisms and also in the search for novel antibacterial agents with the potential application of some major or minor constituents alone, mixture of the extract or in combination with antibiotics for the treatment and prevention of pathologies associated with multi resistant bacteria. However, the mechanism of inhibitory effects of these plant's oils against infectious bacteria is still unclear. Further investigations regarding the in vitro and in vivo should be conducted in order to clear mechanisms pathway and develop such products.

REFERENCES


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UPCOMING CONFERENCES

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