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Al-Ameen College of Pharmacy,
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BRINGING GLOBAL MEDICINAL PLANT RESEARCHERS TOGETHER: PHCOG.NET

PHARMACOGNOSY MAGAZINE
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Prof. B. G. Shivananda Mueen Ahmed K. K.
Director, Phcog.net & Project Co-ordinator, Phcog.net &
Editor, Phcog Magazine Associate Editor, Phcog Magazine
Dept. of Pharmacognosy, Dept. of Pharmacognosy,
Al-Ameen College of Pharmacy, Al-Ameen College of Pharmacy,
Hosur road, Bangalore, Karnataka, India Hosur road, Bangalore, Karnataka, India
Ph: 0091-80-22234619 Ph: 0091-9845655732:
Fax: 0091-80-22225834 E.mail: mueen.ahmed@phcog.net

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Prof. B.G. Shivananda,
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PHCOC MAG.: Peer review Process

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Prof B. G. Shivananda
Editor-in-Chief,
PHCOC MAG.
Dept. of Pharmacognosy,
Al-Ameen College of Pharmacy,
Hosur road, Bangalore, Karnataka,
India

Mueen Ahmed K K
mueen.ahmed@phcog.net
Arun Kumar H .S
arun.kumar@phcog.net

Associate Editors,
PHCOC MAG.
Drug Discovery in Ayurveda - different ways of knowing

Ayurveda is one of the ancient systems of the world. There is no denying the benefits of Ayurvedic treatments that several Indians and others across the globe have experienced. The diagnostic and treatment procedures used are unique and are still valid today as are its foundational principles of panchamahabhutha (five basic elements of nature), tridosha (three humours) and prakriti (individual constitution).

How many of us have stopped to think how the traditional medicines developed. How did the great saints/scholars learn of the medicinal properties of the natural raw drugs that are documented in materia medica? There were no sophisticated modern scientific instruments or facilities available then, which means that there were alternative ways of drug discovery that developed independently of modern western science.

As per Ayurveda, every material (dravya) is a manifestation of five elements (earth, water, fire, air and space) in different proportions. The material could be living as well as non-living things. Depending on the predominant combination of the elements, nature can be categorized into three doshas, namely vata, pitta and kapha. The doshas in humans determines the prakriti, which remains unchanged life-long. Any vitiation from one’s own regular doshic nature leads to ill-health. This vitiation can in turn be set back in balance by drugs, diet and other activities that have the opposite qualities. E.g., kapha dosha is vitiated in a person suffering from cold, which can be balanced by kapha hara (kapha destroying) drug such as Tulsi (Ocimum sanctum). There can be other kapha hara drugs like ginger, turmeric, pepper etc. that can also be used with the same effect. i.e., one is not restricted to a single drug.

So far so good, but then how does one select a particular herb for a specific purpose? It gets tricky at this point! Traditionally, there were trained scholars, called aptas, who conducted experiments on self and observed the effect of every material on the physiology. The drugs were classified as per their rasa (taste), guna (qualities), virya (potency), vipaka (post-digestive effect) and karma (action). There are at least 500 medicinal plant drugs in Ayurvedic materia medica for which every single one of the above information is available.

The parameters used to test and classify each drug in Ayurveda ranged from tasting (sweet, sour, salty, pungent, bitter and astringent) the drug to experiencing its physiological action on the body. This indicates the forethought that has gone into linking pharmacognosy and pharmacology of drugs as inseparable part of the drug. The scholars even went a step further to predict the action of a drug from its taste and other properties. Sweet substances have a tissue-building (brhmana) action, astringent ones have a vaso-constrictive (srotho akunchana) action and so on. Rasa (taste) thus was an important clue to the Ayurvedic way of drug discovery as well as to quality checking.

Till the 18th Century there were new drugs that were added to the materia medica such as Aloe vera and pineapple. However today, the exact science or protocol to test these parameters are not readily available nor understood. Due to this, modern parameters and standards are being used to screen/test Ayurvedic medicines, which do not truly reflect its safety or efficacy. These include phytochemical, anatomical or molecular standards. Unlike in Ayurveda, which used human body and perception to study the properties and action of a drug, there is no single instrument in modern S & T that can check at once the pharmacognosy and pharmacology of the drug. Human sensory evaluation is of particular value in the Food & Beverage industry (such as wine and tea) but not in the modern pharma sector. Sensory evaluation is still used by the traditional Ayurvedic drug industries for raw drug identification and preparation of formulations, but protocols are not documented and therefore appear subjective.

It is worthwhile reviving traditional way of drug discovery and quality control using the human body and senses as the instrument. With concerted efforts at identifying and standardizing the Ayurvedic protocols, we could have an indigenous solution to drug discovery and meaningful quality control standards for Ayurvedic and other traditional medicines.

3. Sharma, PV, Dravyaguna vijnana, Caukamba Bharathi Academy, Varanasi, 2001

Dr. Padma Venkatasubramanian
Joint Director
Foundation for Revitalisation of Local Health Traditions
74/2, Jarakabhande Kaval
Attur Post, via Yelahanka
Bangalore 560 064
Tel: 080 28565618/28566000
Email: padma.venkat@frlht.org
PHCOG MAG.: General Article

Herbal Supplements: Regulation and Safety Aspects
Sunil Kumar*, Dinesh Kumar and Om Prakash

Department of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra-136119
*Corresponding Author: Ph. 09416581005 ; E-mail: sunilmadhuban@yahoo.com

ABSTRACT - Many people all over the world use herbal supplements but there is no strict regulation on these products. Before 1994, these were regulated as food and were evaluated for safety before being launched into the market for consumer purchase. In 1994, Dietary Supplement Health and Education Act (DSHEA) was passed which weakened the FDA and allowed the distribution herbal supplements without testing their efficacy and toxicity. Currently, there are many agencies, which are working on the regulation of these products. Herbal supplements may also cause toxic reactions. So, people should be aware about the safety and adverse effects of these products.

KEYWORDS: Herbal Supplements, FDA, DSHEA, GMP, German Commission E, WHO.

INTRODUCTION
Herbal supplements (a type of dietary supplement) are simple or multicomponent herb mixture used to supplement the traditional medical treatment. Dietary supplement is a product other than tobacco intended to enrich the diet containing one or more of vitamins; minerals; herbs or other botanicals; amino acids; or any combination of the above ingredients and is not used as a conventional food or as a sole item of a meal or the diet (1).

Regulation of herbal supplements
In the early 20th century, fraud was rampant among the producers of both food and drugs in the United States (2). There was no regulating body or committee to check fraudulent practices such as misbranding and adulteration of drugs, so in order to ensure the identity, purity, quality, strength, composition, safety and effectiveness of dietary supplements, the following organizations came into existence for their regulation:

Food and Drug Administration (FDA)
The act to create this agency was passed in 1906 by efforts of Harvey W. Wiley, then chief of the Bureau of Chemistry of the U.S. department of agriculture (2). The job of the FDA is to regulate the drugs, food, and cosmetics in several countries, which assures the public that drugs are safe and effective and have been subject to scientific scrutiny. In 1962, the FDA required that all drugs be evaluated for safety and efficacy (3).

It is estimated that more than 1400 herbs are commonly sold and promoted for medicinal uses worldwide (4,5). The FDA maintains a list of products “Generally Recognized as Safe” (GRAS). Approximately 250 herbs appear on this list, but these are herbs used for food flavoring and not for medicinal purposes. Currently, only a handful of herbs have been shown safe and effective based on a 1990 FDA review of over-the-counter drugs (6).

In 1993, the FDA distributed an advance notice of a proposed rule that addressed the concerns regarding the herbal and supplement industry. The report discussed instances of herb-related deaths and concerns about toxicities. But the manufacturers had little incentive to seek FDA approval due to the costs associated with drug research and to avoid the burden of proof associated with FDA approval, herbal manufacturers began to label herbs as “foods” and sell them in health food stores. Due to public and supplement industries concerns, DSHEA was constituted, which limits the FDA’s influence on herbal products (5). Now FDA can only take action if a product is found to present a significant or unreasonable risk of illness or injury (7).

Dietary Supplement Health and Education Act (DSHEA)
The act to create this agency was passed in 1994 and it supported the fact that dietary supplements (which include herbal medicines) can play an important role in health promotion and the prevention of chronic disease (8,9). This legislation allows herbal products to be sold without testing for efficacy. The herbal supplements manufacturing companies cannot make claims on an herb’s ability to cure a disease, but they may claim about how a supplement affects the structure and function of the body (10). Under this act, herbs can be sold without FDA approval (4-5,10-12). The only way a dietary supplement can be withdrawn from the consumer market is if the FDA can prove that the
product is unsafe (1,7,13). This legislation defined the dietary supplements, addressed safety issues and provided a mechanism for monitoring safety. DSHEA also called for good manufacturing practices to be used in producing dietary supplements.

**World Health Organization (WHO)**

World Health Organization's Guidelines for the Assessment of Herbal Medicines, which state that a substance's historical use is a valid way to document safety and efficacy in the absence of scientific evidence to the contrary (14). A long history of use may allow for safety information to be gathered; however, it may do little to assess efficacy.

**German Commission E**

German Commission E was established in 1978. It is the interdisciplinary commission of scientists and health professionals, which is responsible for reviewing herbal medicines. In its review, the commission considers traditional use; chemical data; clinical, experimental, pharmacological, toxicological and epidemiological studies; patient case records from physicians' files; and unpublished proprietary data from manufacturers to determine the safety and effectiveness of each herbal medicine. It then develops monographs for informing the public of its findings. These monographs have recently been translated into English and are useful tools for other countries attempting to establish high standards for herbal medicines (7,15).

This Commission E has reviewed clinical literature (including clinical trials and case studies) on more than 1400 herbal drugs (4,5). The commission has produced more than 300 monographs on common herbal remedies. However, these monographs must be used with caution given their reliance on historical bibliographic information that may or may not include data gathered from clinical trials (16).

**Labeling Requirements**

FDA requires that certain general information must appear on the dietary supplement label:

- Name of product (including the word “supplement” or a statement that the product is a supplement)
- Net quantity of contents
- Name and place of business of manufacturer, packer, or distributor
- Directions for use

The label of a dietary/herbal supplement product is required to be truthful and not misleading. If the label does not meet this requirement, FDA may remove the product from the marketplace or take other appropriate actions.

A label may not claim that a product can be used to diagnose, treat, cure, or relieve a specific disease. For example, it cannot include the claim “treats arthritis.” Regulations apply only to product labels. However, unapproved health claims can appear in places other than the label, such as magazines, websites, and signs in stores.

**Good manufacturing practices (GMP) Regulation**

FDA is authorized to issue Good Manufacturing Practice (GMP) regulations describing conditions under which dietary supplements must be prepared, packed, and stored. DSHEA also maintains the FDA’s right to establish good manufacturing practices (GMPs), which are standard for the pharmaceutical industry and felt to be a key to product purity and safety (10). FDA published a proposed rule in March 2003 that is intended to ensure that manufacturing practices will result in an unadulterated dietary supplement and that dietary supplements are accurately labeled. Until this proposed rule is finalized, dietary supplements must comply with food GMPs, which are primarily concerned with safety and sanitation rather than dietary supplement quality. Some manufacturers voluntarily follow drug GMPs, which are more rigorous, and some organizations that represent the dietary supplement industry have developed unofficial GMPs. These regulations would govern the preparation, packing, and holding of dietary supplements under conditions that assure their safety. These regulations are to be modeled under guidelines currently in effect for the food industry. To date, the FDA has not fully implemented manufacturing guidelines for the herbal industry (17). The herbal industry has taken strides to police itself with regard to product quality. The National Nutritional Foods Association randomly tests products produced by its members. The Association also plans to begin certification of factories every three years using the same good manufacturing processes proposed by the FDA, although manufacturers are not obligated to belong to this organization. In addition to the National Nutritional Foods Association, the United States Pharmacopoeia (USP) sets standards for pharmaceuticals, vitamins, and minerals. The USP, a private, nonprofit organization, has begun to produce monographs about herbs that sum up evidence of effectiveness and detail standards for quality, strength, and purity of the final product (17). Adoption of these standards is voluntary,
and manufacturers claiming to meet them are not checked except in response to complaints.

**Safety and Purity**

The prevalence of herbal use is largely unstudied (4,5,18). Also, the lack of scientific research on herbs, combined with the lack of FDA regulation of herbal preparations, can give consumers a false sense of security about the safety of herbal supplements (11,19,20). Both consumers and health care professionals are concerned about whether herbal products are safe. Sometimes, even if you take an herb or supplement for one certain reason, there can be other unintended reactions. Herbal and dietary products have chemical properties just like manufactured drugs. So, these may also have side effects. One of the major problems with many of the products on the market today is that the amount and the purity of their active ingredients vary so greatly from product to product. In many cases, we do not always know how much of the natural substance we are really getting in each dose or if other ingredients have been added. Another problem is determining how much of each active ingredient is really safe, particularly over long-term use. There are even case reports of contaminated herbs causing death. Also, studies are being done to see how herbs and supplements react with other medications.

The dose and form of a botanical preparation also play important roles in its safety. Teas, tinctures, and extracts have different strengths. The same amount of a botanical may be contained in a cup of tea, a few teaspoons of tincture, or an even smaller quantity of an extract. Also, different preparations vary in the relative amounts and concentrations of chemical removed from the whole botanical. The safety of herbal products may be related to the mixtures of active chemicals that they contain; their interactions with other herbs and drugs, contaminants, or adulterants; or their inherent toxicity. Active ingredients in herbs and dietary supplements can cause unexpected reactions when used with other herbs or medications. Effects on the distribution, metabolism, or excretion of drugs may be pronounced and may lead to drug toxicity. Contaminants and adulterants of herbal products can be pharmacologically active and responsible for unexpected toxicity. Because of the variability in herbal product ingredients, the actual dose of active ingredients being consumed is often variable, unpredictable, or simply unknown. When compared with adults, children may be particularly susceptible to the effects of such dosage variations by virtue of their smaller size and different capacity for detoxifying chemicals.

Nearly 16% to 18% of adults in the United States regularly use herbal supplements (21). Sales of herbal products in the United States doubled to $16 billion between 1994 and 2002 and 23% of those >50 years use herbal products (22). There are about 15 million adults at risk of experiencing adverse interactions from prescription medication, herbs, and/or vitamin supplements, including nearly three million adults age 65 or older (19,23,24). Given the misconception that herbal supplements have benign side effects, it is not surprising that one study found that almost two thirds of patients do not tell their physicians that they are taking supplements (25,26). Therefore it is imperative to ask each patient to tell his health care provider every medication (prescribed and over-the-counter) as well as every vitamin and supplement that they are taking or have been taking recently. The physician should warn patients that such supplements may interfere with prescribed medications or that they may have side effects.

Numerous cases of toxicity have been linked to the use of herbal products (Table 1). The resulting problems range from minor adverse reactions to serious physical disabilities and death. The herb ma huang and all ephedrine alkaloids have received considerable attention from the FDA. More than 15 deaths have been attributed to the use of ephedrine alkaloid products (27). In 1996, the FDA issued a warning to consumers to avoid nutritional supplements containing ephedrine (28). In 1997, the FDA proposed the use of warning labels addressing the adverse effects of ephedrine, banning products containing more than 8 mg per serving, and eliminating products containing combinations of ephedrine and caffeine (29). A recent FDA report identified 76 botanicals known or suspected of containing aristolochic acid and 92 botanicals believed adulterated with aristolochic acid. Products containing a large amount of this substance may produce rapid-onset toxicity. However, the effects of long-term use are unknown. The first indication of adverse effects may be irreversible, such as renal failure (30).

Despite safety claims, patients and health care providers should be aware that abuse of dosages and problems with adulteration may render an otherwise safe herbal product dangerous. Ginseng, although considered by many sources to be relatively safe, had a
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In June 2000, the New England Journal of Medicine published a report by Nortier and others of an outbreak of urinary tract cancers in Belgium among users of a Chinese herbal product that contained aristolochic acid -- a known carcinogen found in an herb called *Aristolochia fangî* (33).

Table I: Side effects of some most commonly used herbal supplements

<table>
<thead>
<tr>
<th>Common Name/ Source</th>
<th>Use</th>
<th>Possible Side effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ginkgo</strong> <em>(Ginkgo biloba)</em></td>
<td>Dementia, memory improvement, SSRI-related impotence, antioxidant, inhibit platelet aggregation</td>
<td>GI upset, headache, Nausea, Vomiting&lt;sup&gt;35&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Kava</strong> <em>(Piper methysticum)</em></td>
<td>Anxiolytic, sedative, muscle relaxant, anti-convulsant</td>
<td>Rare skin rash, sedation, Sedatives, hepatotoxicity&lt;sup&gt;36, 37&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>St. John’s Wort</strong> <em>(Hypericum perforatum)</em></td>
<td>Antidepressant, MAO inhibitor&lt;sup&gt;38&lt;/sup&gt;</td>
<td>Photodermatitis, GI upset, sedation, Restlessness, fatigue, phototoxicity&lt;sup&gt;39&lt;/sup&gt; constipation, dizziness, dry mouth, confusion&lt;sup&gt;37, 40, 41&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ephedra</strong> <em>(Ephedra spp.)</em></td>
<td>Stimulant, nasal Decongestant, Bronchodilatior, appetite suppressant&lt;sup&gt;42&lt;/sup&gt;</td>
<td>Death in overdose, cardiovascular Complications, Seizures, high blood pressure, cardiac arrhythmia and infarction, insomnia, psychosis, stroke, urine retention, uterine contractions&lt;sup&gt;42&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ginseng</strong> <em>(Panax ginseng)</em></td>
<td>CNS stimulation and suppression, hypertensive, hypoglycemic, antioxidant, anti-inflammatory, anticancer, platelet inhibition, immune stimulant, antifatigue, improve sexual functions&lt;sup&gt;37&lt;/sup&gt;</td>
<td>Diarrhea, euphoria, headache, hypertension, hypotension, insomnia (relatively common), mastalgia, nausea, vaginal bleeding, Sleeplessness, nervousness, hypertension, euphoria (GAS); hypertension together with nervousness, sleeplessness, skin eruptions, edema, morning diarrhea&lt;sup&gt;32, 37, 44, 45&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Echinacea</strong> <em>(Echinacea spp.)</em></td>
<td>Immune system stimulant, antifungal, anti-inflammatory</td>
<td>Anaphylaxis (rare)&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Kava</strong> <em>(Piper methysticum)</em></td>
<td>Anxiolytic, muscle relaxant, mood enhancer, analgesic, sedative, antibacterial, platelet inhibitor, sedative</td>
<td>Reversible discoloration of skin, nails and hair&lt;sup&gt;46&lt;/sup&gt; (chronic abuse); visual disturbances; dizziness; stupor; gastrointestinal discomfort; extrapyramidal effects&lt;sup&gt;47&lt;/sup&gt; (rare); hepatotoxic&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Saw palmetto</strong> <em>(Serenoa repens)</em></td>
<td>Treat benign prostatic hyperplasia, Improve overall prostate health, Enhance sexual vigor, enhance breast size.</td>
<td>GI disturbances, headaches, Large amounts may cause diarrhoea&lt;sup&gt;37&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Aloe (Aloe barbadensis)  Laxative  Loss of electrolytes with chronic use\textsuperscript{48}

Cascara (Rhamnus purshiana)  Topical analgesic/counter irritant  Loss of electrolytes with chronic use\textsuperscript{48}

Senna (Cassia spp.)  Laxative  Diarrhea, nausea; avoid chronic use.\textsuperscript{48}

Witch hazel (Hamamelis virginiana)  Astringent  Stomach irritation, liver damage if taken internally (rare).\textsuperscript{48}

Valerian root (Valeriana officinalis)  Sleep aid\textsuperscript{49}  Decreases blood pressure, heart palpitations, upset stomach\textsuperscript{50}

Feverfew (Crysanthemum parthenium)  Treatment of migraine headaches, anti-inflammatory\textsuperscript{51}  Increases heart rate, allergic reactions, mouth ulcers, headaches, gastric disturbances, post feverfew syndrome (withdrawal symptoms of aches, pains, and joint and muscle stiffness)\textsuperscript{52}

Evening primrose oil (Oenothera spp.)  Anti-inflammatory, sedative, anticoagulant, astringent  Gastrointestinal disturbances\textsuperscript{53}

<table>
<thead>
<tr>
<th>Common name/ Source</th>
<th>Prescribed drugs</th>
<th>Possible interaction</th>
</tr>
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<tbody>
<tr>
<td><strong>Ginkgo</strong> (Ginkgo biloba)</td>
<td>Aspirin</td>
<td>Increased anti-coagulation\textsuperscript{55}</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>Haemorrhage\textsuperscript{56}</td>
</tr>
<tr>
<td></td>
<td>Warfarin</td>
<td>Potentiation\textsuperscript{57}</td>
</tr>
<tr>
<td><strong>Ginseng</strong> (Panax ginseng)</td>
<td>Phenelzine</td>
<td>Headache, tremor, mania\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Antidepressants</td>
<td>Induces mania in depressed patients\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Benzodiazapines</td>
<td>Possible additive effects\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>MAOI’S</td>
<td>Possible additive effects\textsuperscript{46}</td>
</tr>
<tr>
<td></td>
<td>SSRI’s (Antidepressant)</td>
<td>Additive serotonin-like effects \textsuperscript{51}</td>
</tr>
<tr>
<td><strong>St. John’ s Wort</strong> (Hypericum perforatum)</td>
<td>Theophylline</td>
<td>Decreased theophylline concentration\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Serotonin-reuptake inhibitors</td>
<td>Mild serotonin syndrome, decreased bioavailability of digoxin, theophylline, cyclosporin, phenprocoumon\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Paroxetine</td>
<td>Lethargy, incoherence\textsuperscript{54} Nausea, Fatigue\textsuperscript{58}</td>
</tr>
<tr>
<td><strong>Liquorice</strong> (Glycyrrhiza glabra)</td>
<td>Hydrocortisone</td>
<td>Glycyrrhetinic acid (an acid in topical anti-inflammatories) potentiates cutaneous vasoconstrictor response\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Oral and topical corticosteroids</td>
<td>Potentiates corticosteroids\textsuperscript{54}</td>
</tr>
<tr>
<td></td>
<td>Oral contraceptives</td>
<td>Hypertension, edema, hypokalemia\textsuperscript{54}</td>
</tr>
</tbody>
</table>
Interaction between herbal supplements and prescribed drugs:
Patients often neglect to mention herbal supplements when asked by their health care providers about medications taken on a regular basis because they assume that herbs are natural and also feel their physician will not approve of their herbal use. However, not informing health care providers about herbal supplements use places patients at risk because of the possible interactions between drugs and herbs (Table 2). The known effects of using prescription drugs and herbs in combination are that herbs can mimic, magnify, or oppose the effect of the drugs. (53-54)

CONCLUSION
Many people use herbal supplements because they believe natural means safer and fewer side effects. The truth is, many herbs are dangerous and can interact with your prescription and over-the-counter drugs. Therefore the public need to be aware that “natural” does not mean “safe.” Herbs should not be considered as miraculous cure-alls but rather compounds that work through simple biochemistry. Specific compounds trigger a specific physiologic effect—an effect that can be exacerbated if too much of a product is used or if it is used in combination with other medications. In addition, public should be aware that the hyperbolic advertising and advocacy literature surrounding herbal products often contains untested claims. If someone wishes to take an herbal supplement, he or she should use a standardized product. Products should have the scientific name and quantity of the botanical clearly identified on the label. The name and address of the manufacturer, lot number, and expiration date should be clearly marked. Inform the doctor, pharmacist and other health care professionals of any herbs we are considering or routinely use. People should be advised to stop taking the herb immediately if adverse effects occur.

REFERENCES
(2000).
PHCOG MAG.: Research Article

*In-vitro* anthelmintic activity of stem bark of *Mimusops elengi* Linn.

Mali R.G. ¹, Mahajan S.G¹ and Mehta A.A.²

¹Department of Pharmacognosy and Phytochemistry, Smt. S.S.Patil College of Pharmacy, Chopda, Maharashtra, India.

²Department of Pharmacology, L.M. College of Pharmacy, Navrangpura, Ahmedabad, Gujarat, India.

Corresponding author: ravigmali@yahoo.co.in

ABSTRACT - The aim of present study was to evaluate anthelmintic potential of crude alcoholic extract of bark of *Mimusops elengi* and its different fractions namely ethyl acetate, n-butanol and methanol using *Pheretima posthuma* and *Ascardia galli* as test worms. Various concentrations (10 - 100 mg/ml) of alcoholic extract and its various fractions were tested in the bioassay, which involved determination of time of paralysis (P) and time of death (D) of the worms. Piperazine citrate (10 mg/ml) was included as standard reference and distilled water as control. The results of present study indicated that the crude alcoholic extract and its ethyl acetate and n-butanol fractions significantly demonstrated paralysis, and also caused death of worms especially at higher concentration of 100 mg/ml, as compared to standard reference Piperazine citrate. In conclusion, the traditional use of bark of the plant *M. elengi* as an anthelmintic have been confirmed and further studies are suggested to isolate the active principle/s responsible for the activity.


INTRODUCTION

*Mimusops elengi* Linn (Sapotaceae) commonly known as Bakul, is a small to large evergreen tree found all over the different parts of India. It is cultivated in gardens as an ornamental tree. It has been used in the indigenous system of medicine for the treatment of various ailments. Several therapeutic uses as cardiotonic, alexipharmic, stomachic, anthelmintic and astringent have been ascribed to the bark of *Mimusops elengi* (1). Phytochemical review shows the presence of taraxerol, taraxerone, ursolic acid, betulinic acid, α-spinossterol, β-sitosterol, lupeol, alkaloid isoretronecylic tiglate and mixture of triterpenoid saponins in the bark of *Mimusops elengi* (2-5). Literature search revealed that there is no report available regarding anthelmintic activity of *M. elengi* bark. The present study was, therefore undertaken to evaluate the *in vitro* anthelmintic activity of crude extract of *M. elengi* bark (70 % alcoholic extract) and its different fractions against *Pheretima posthuma* and *Ascardia galli*.

MATERIALS AND METHODS

*Plant Collection and Authentication*

The stem bark of *M. elengi* was collected from mature trees and its botanical identification was confirmed from Botanical Survey of India (BSI), Koregaon Road, Pune. A voucher specimen RGM-A2 was deposited in the herbarium of BSI, Pune.

*Plant Extraction*

The plant material (stem bark) was dried for several days and powdered with the help of an electric grinder. After defatting the bark powder (250 g) using petroleum ether (40 - 60 °C), it was air dried and extracted exhaustively with 70 % alcohol. The liquid extract was evaporated in vacuum to yield 34.5 % dark brown residue. The dried residue was taken in minimum quantity of water and was successively extracted with ethyl acetate, n-butanol and methanol to yield 4.92 % w/w, 7.85 % w/w and 8.20 % w/w residue, respectively. Alcoholic extract and all the fractions were preserved in refrigerator.

*Worms Collection and Authentication*

Indian earthworm *Pheretima posthuma* (Annelida) were collected from the water logged areas of soil and *Ascardia galli* (Nematode) worms were obtained from freshly slaughtered fowls (*Gallus gallus*). Both worm types were identified at the P.G. Department of Zoology, Pratap College, Amalner.

*Preparation of Test Sample*

Samples for in-vitro study were prepared by dissolving 2.5 gm of each crude alcoholic extract and its ethyl acetate, n-butanol and methanol fractions in 25 ml of
distilled water to obtain a stock solution of 100 mg/ml. From this stock solution, different working dilutions were prepared to get concentration range of 10, 50 and 100 mg/ml.

**Anthelmintic Assay**

The anthelmintic assay was carried as per the method of Ajayeeoba E.O. *et al* (6) with minor modifications. The assay was performed on adult Indian earthworm, *Pheretima posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings (7-10). Because of easy availability, earthworms have been used widely for the initial evaluation of anthelmintic compounds in vitro (11-14). *Ascardia galli* worms are easily and plentifully available from freshly slaughtered fowls and its use, as a suitable model for screening of anthelmintic drug was advocated earlier (15-17). 50 ml formulations containing three different concentrations, each of crude alcoholic extract and its various fractions (10, 50 and 100 mg/ml in distilled water) were prepared and six worms (same type) were placed in it. This was done for both types of worm. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water (50 °C) (18-19). Piperazine citrate (10 mg/ml) was used as reference standard while distilled water as the control.

**Table 1: Anthelmintic activity of alcoholic extract of *Mimusops elengi* and its fractions**

<table>
<thead>
<tr>
<th>Test subs</th>
<th>Concentration mg/ml</th>
<th>Time taken for Paralysis (P) and Death (D) of worms in minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. posthuma</em> (P)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>25 ± 0.2</td>
</tr>
<tr>
<td>Alcoholic extract</td>
<td>50</td>
<td>18 ± 0.1***</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>12 ± 0.3***</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>10</td>
<td>26 ± 0.3</td>
</tr>
<tr>
<td>fraction</td>
<td>50</td>
<td>20 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>13 ± 0.5***</td>
</tr>
<tr>
<td>n - Butanol fraction</td>
<td>10</td>
<td>27 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>19 ± 0.2**</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>14 ± 0.1***</td>
</tr>
<tr>
<td>Methanol fraction</td>
<td>10</td>
<td>28 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>25 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20 ± 0.4</td>
</tr>
<tr>
<td>Piperazine citrate</td>
<td>10</td>
<td>23 ± 0.8</td>
</tr>
</tbody>
</table>

All values represent Mean ± SEM; n=6 in each group. Values are significantly different from reference standard (Piperazine citrate) *p<0.05; **p<0.01; ***p<0.001

**RESULTS AND DISCUSSION**

Preliminary phytochemical screening of crude alcoholic extract and its different fractions revealed the presence of alkaloids, saponins, flavonoids and tannins. As shown in Table 1, alcoholic extract of *M. elengi* and its different fractions exhibited anthelmintic activity in dose-dependant manner giving shortest time of paralysis (P) and death (D) with 100 mg/ml concentration, for both types of worms. The alcoholic extract of *M. elengi* caused paralysis of 12 min and time of death of 29 min while ethyl acetate and n-butanol fractions revealed paralysis of 13 and 14 min. and time of death of 31 and 30 min. respectively against the earthworm *P. posthuma*. The reference drug Piperazine citrate showed the same at 23 and 60 minutes, respectively.

*Ascardia galli* worms were also shown sensitivity to the alcoholic extract and different fractions significantly higher concentration of 100 mg/ml. The alcoholic extract caused paralysis at 8 min. and time of death of 18 min. Ethyl acetate and n-butanol fractions showed paralysis at 9 and 11 min and the time of death were 19 and 21 min. respectively. Piperazine citrate exhibited similar effects at 16 and 33 min. respectively. The predominant effect of Piperazine citrate on worm is to cause a flaccid paralysis those results in expulsion of the worm by peristalsis.
Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyperpolarisation and reduced excitability that leads to muscle relaxation and flaccid paralysis (20). The stem bark extract of M. elengi not only demonstrated paralysis, but also caused death of worms especially at higher concentration of 100 mg/ml, in shorter time as compared to reference drug Piperazine citrate. Phytochemical analysis of the crude extracts revealed the presence of tannins among the other chemical constituent contained within them. Tannins were shown to produce anthelmintic activities (21). Chemically tannins are polyphenolic compounds (22). Some synthetic phenolic anthelmintics e.g. niclosamide, oxyclozanide, bithionol etc., are reported to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (23). It is possible that tannins contained in the extracts of M. elengi produced similar effects. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal (24) or glycoprotein on the cuticle of the parasite (25) and may cause death.

The traditional medicines hold a great promise as source of easily available effective anthelmintic agents to the people, particularly in developing countries, including India. It is in this context that the people consume several plants or plant-derived preparations to cure helminthic infections (26). The origin of many effective drugs has been found in the traditional medicines practices and in view of this it is important to undertake studies pertaining to screening of the folklore medicinal plants for their proclaimed anthelmintic efficacy.

In conclusion, the traditional use of stem bark of Mimusops elengi as an anthelmintic have been confirmed as the stem bark extracts displayed profound arithmetic activity in the study. Further, it would be interesting to isolate the possible phyto constituents which may be possible responsible for the anthelmintic activity and to possible the mechanism (s) of action.

REFERENCES
PHCOG MAG.: Research Article

Pharmacological effects of *Trigonella foenum graecum* Linn. seeds on various isolated perfused smooth muscle preparations

Natarajan B. and Dhananjayan. R

Department of Pharmacology and Environmental Toxicology, Dr. A.L.M. P.G. Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai – 600 113. Tamil Nadu, India.

E-mail: natarajanphd@yahoo.com

ABSTRACT - In the present study, the pharmacological effects on different smooth muscles by the extracts like total alcoholic (TA), total aqueous (TQ), petroleum ether (PE), total alkaloidal (TK), total glycosidal (TG) and a phytochemical trigonelline (TR) from *Trigonella foenum graecum* Linn. seeds was investigated using isolated organ bath preparation method. 1 mg/ml dose of the extracts and the phytochemical showed significant anti-histaminic activity on guinea pig ileum, anti-cholinergic effect on rat colon and uterine stimulant activity on rat uterus. These results might be due the presence of some spasmylytic and spasmogenic constituents in the *Trigonella foenum graecum* Linn. (Fenugreek) seeds. These findings will help to invent some novel therapeutics from the fenugreek seeds.

KEYWORDS: *Trigonella foenum graecum*, total alkaloidal, total glycosidal, petroleum ether, ileum, spasmylytic, spasmogenic.

INTRODUCTION

*Trigonella foenum graecum* Linn. belongs to the family Leguminosae and it is popularly known as Fenugreek (1). *Trigonella foenum graecum* Linn. is native to the area from the Eastern Mediterranean to Central Asia and Ethiopia, and much cultivated in India and China(2). The seeds of *Trigonella foenum graecum* Linn. is the main focus of this study. Fenugreek is one such plant whose seeds and leaves are used not only as food but also as an ingredient in traditional medicines (3). In India, the seeds of fenugreek were used in Ayurveda and Siddha to treat fever, dysentery and heart diseases, while in Unani system, this plant is used as a resolvent, aphrodisiac, diuretic, emmenagogue and tonic (4). In China, fenugreek seeds were used as a galactogogue to encourage lactation (5). The past phytochemical investigations on the seeds reveals the presence of Diosgenin, Trigonelline, Gitogenin, Vicenins 1 and 2, Vitexin, Quercetin, Luteolin, Kaempferol, β-Sitosterol etc., moreover the endosperm of the seeds is rich in galactomannan (6). In our previous study, for the first time we have proven that the seeds possess cardiotonic property (7). Also the seeds were shown to possess various pharmacological properties like gastric anti-ulcer activity (8), wound healing activity (9) and immuno modulatory activity (10).

Several synthetic spasmylegens and spasmylytics have been used in the field of medicine, in spite of their side effects and minimum therapeutic index. Even though many drugs are available with spasmylegenic and spasmylytic properties, search for a drug of plant origin with maximum potency and minimum side effects continues. Since no scientific report of the previous investigations detail are available on the effect of fenugreek on the isolated smooth muscles, the present study was undertaken to screen the extracts and a phytochemical of fenugreek on various smooth muscle preparations using isolated organ bath preparations.

MATERIALS AND METHODS

Preparation of extracts

Fenugreek seeds were obtained from the supermarket and authenticated by a botanist Prof. Dr. R. Rengasamy, C.A.S. Botany, University of Madras. A voucher specimen of the sample has been deposited in the department of Pharmacology and Environmental Toxicology, Dr. A.L.M. P.G.IBMS, University of Madras, Chennai.

Total alcoholic extract

500 g of dried and coarsely powdered seeds of *Trigonella foenum graecum* Linn. was extracted with 95% ethanol for a period of one month. The filtrate
was taken and concentrated on a water bath using petridish. The temperature was maintained at 55°C. The concentrate obtained was weighed (25 g) and coded as TA.

**Total aqueous extract**

500 g of dried and coarsely powdered seeds of *Trigonella foenum graecum* Linn. was extracted with double distilled water for 24 hrs. The filtrate was taken and concentrated on a water bath using petridish. The temperature was maintained at 60°C. The concentrate obtained was weighed (16.4 g) and coded as TQ.

**Petroleum ether extract**

500 g of the dried and coarsely powdered seeds were extracted with petroleum ether and kept at room temperature, same as TA. The filtrate was obtained and evaporated to a concentrate using petridish on water bath. Concentrate obtained was weighed (25.6 g) and coded as PE.

**Total alkaloidal extraction**

10 g of total alcoholic extract concentrate was extracted with 0.1N HCl by allowing it to stand for 5 hrs. The aqueous acid extract was partitioned with 100 ml of chloroform in a separating funnel. This procedure was repeated for 2 more times and the combined chloroform layer was rejected. The aqueous layer was basified with ammonium hydroxide to pH 9.0 and was again partitioned with chloroform. The aqueous layer was rejected while the chloroform layer was collected and evaporated to obtain the concentrate (11). The total yield was 200 mg and it was coded as TK.

**Total glucosidal extraction**

500 g of shade dried and coarsely powdered seed of *Trigonella foenum graecum* Linn. was extracted with ethanol: water (1:2). The aqueous alcoholic extract thus obtained was treated with 5% neutral lead acetate to precipitate the tannins present. This procedure was repeated until no more precipitate of lead tannate was obtained which was filtered off. The clear filtrate was bubbled with H₂S (Hydrogen sulphide gas) to remove the excess lead present in the solution as black lead sulphide. The black precipitate was filtered and the process was repeated until no more black precipitate of black lead sulphide was obtained or the filtrate smells strongly of H₂S. The clear filtrate was evaporated to a concentrate (12). The total yield was 22 g and it was coded as TG.

**Identification of Trigonelline in the alcoholic extract of fenugreek seeds**

Previous literature study reveals that fenugreek seeds contain Trigonelline. The separation and purification of Trigonelline would be a demanding, time consuming and expensive work. Instead of isolating the Trigonelline, it was obtained of pure grade from Sigma Chemical Co., USA and subjected to TLC for identification. The Trigonelline obtained was used for the pharmacological studies.

The alkaloid Trigonelline was identified in the total alcoholic extract using thin layer chromatography (TLC) by comparing it with the standard trignonelline of Sigma grade. TLC provided a chromatographic drug fingerprint. Silica gel 60 F₂₅₄ precoated TLC plates were used. The solvent system used for Trigonelline is Methanol:Ammonia (200:3) (13). Detection was done by placing the TLC plate in the ultraviolet chamber for Trigonelline. Fig.1 shows the presence of trignonelline in the total alcoholic extract when compared with the Trigonelline standard. All the experiments carried out in this study were approved by Institutional Animal Ethical Committee (IAEC).

**Isolated perfused guinea pig ileum preparation**

Guinea pigs weighing about 200 - 300 g were used for the ileum experiment. This tissue proves to be a classical model for the bioassay of histamine. H₁ receptor mediated responses were studied in guinea pig longitudinal smooth muscle of the ileum (14). Ileum was removed from the guinea pig and placed in a petridish containing Tyrode solution (Composition in mM: NaCl - 136.89, KCl - 2.68, MgCl₂ - 1.05, CaCl₂ - 1.36, NaH₂PO₄ - 0.32, NaHCO₃ - 11.90 and Glucose - 5.55; made up to 1 litre of distilled water). Ileum was cut into small pieces and 2 pieces of threads were tied at the upper and lower ends of an ileal tissue.
The tissue was mounted in a micro organ bath of capacity of 10 ml, which was maintained at 37°C, aerated and perfused with Tyrode solution. The tied ileal tissue was attached through a thread to a frontal lever balanced at a tension of 0.5 g. The writing arm of the frontal lever was made to touch the kymograph drum. The drugs were administered in the organ bath containing the tissue and the response was recorded on the kymograph. The following cycle was adopted:
0 sec - the kymograph was switched on
15 sec - the standard drug was added
45 sec - the test compound was added
75 sec - the kymograph was switched off and the preparation was washed with a fresh change of perfusion fluid (15).

**Isolated perfused rat colon preparation**

Matured rats weighing about 200 - 250 g were used for this experiment. Rat colonic muscle is the best model for evaluating the muscarinic or anti-muscarinic effect of a test drug. Biochemical and Northern blot studies have shown that both muscarinic M2 and M3 receptors are present in mature rat colon (16). The first 4 cm of the descending colon was used in this experiment.

The colon was isolated from the rat and placed in a petridish containing Modified Ringer’s solution (Composition in mM: NaCl - 171, KCl - 5.36, NaHCO₃ - 1.7, CaCl₂ - 0.270, Glucose - 5.55). The tissue was mounted in the micro organ bath perfused with Modified Ringer’s solution that was maintained at 32°C, aerated and the contractions were recorded as described for the previous experiment.

**Isolated perfused rat uterus preparation**

The uteri were obtained from the virgin female albino rats pretreated with estradiol benzoate (0.5 mg/kg b.w, s.c) (17), 24 hrs prior to the experiment. The presensitized uterus of the rat has been used widely for evaluating the uterine stimulant/tocolytic activity of drug/extracts. The two horns of the uterus were placed in the petridish containing De Jalon’s solution (composition in mM: NaCl - 154, KCl - 5.6, NaHCO₃ - 5.95, CaCl₂ - 2.2, Glucose - 2.8). A single horn was mounted in the micro organ bath of 10 ml capacity, which was maintained at 30°C, aerated and perfused with De Jalon’s solution. The contractions were recorded as described for the previous experiment.

**Statistical analysis**

Six tissues from different animals were used for each series of experiment. The data were analyzed using the student’s t - test. The values are expressed as mean ± S.E.M.

**RESULTS**

**Isolated perfused guinea pig ileum preparation**

The anti-histaminic activity of the extracts and the phytochemical of fenugreek seeds were observed. Fig. 2 reveals the response obtained from the extract and the phytochemical on guinea pig ileum. Histamine (1 µg) produced a contractile effect on the ileum. The alkaloidal extract (1 mg/ml) - TK and trigonelline (1 mg/ml) - TR produced significant blocking effects on the histamine induced contraction. The code TK showed 45.76% of antagonistic effect and the code TR showed 50.17% of antagonistic effect.

**Isolated perfused rat colon preparation**

The extracts and the phytochemical were analyzed for the anti-cholinergic effects. Fig. 3 reveals the anti-cholinergic activity of the extracts. Acetylcholine (1 µg) produced contraction on the rat colon muscle. The total aqueous (1 mg/ml) - TQ and the total glycosidal (1 mg/ml) - TG extracts showed significant antagonistic effect on the acetylcholine induced contraction. The code TQ showed 39.03% and code TG showed 43.91% blocking effect respectively.

**Isolated perfused rat uterus preparation**

The extracts produced significant contraction on the isolated rat uterus. Oxytocin (0.1 IU) and acetylcholine (1 µg) possessed significant contraction on the uterus muscle. Table 1. reveals the utherotonic effect of the alcoholic (1 mg/ml) - TA, aqueous (1 mg/ml) - TQ and petroleum ether (1 mg/ml) - PE extracts as same as the oxytocin, which was confirmed by the blocking effect of indomethacin (10 µg). Oxytocin produced 5.88 cm contraction, acetylcholine produced 3.13 cm contraction, while the codes TA produced 4.21 cm, TQ produced 3.96 cm and PE produced 3.98 cm respectively.

**DISCUSSION**

In various indigenous systems of medicine, the seeds of *Trigonella foenum graecum* Linn. were used. The extractions of seeds in previous research work were done in a rambling manner and some pharmacological investigations were done by using only alcoholic and aqueous extracts, moreover there was no evidence of isolation of alkaloids and glycosides from this seeds. So, it was decided to do the systematic extraction, which helped to compare the pharmacological potentials of different extracts on guinea pig ileum, rat colon and rat uterus muscles.

In guinea pig ileum, the longitudinal smooth muscle contains histamine receptors of both H₁ and H₂ types, with the former being responsible for the contractile response to histamine (18). The contractile response obtained with 1 µg of histamine was considered as
Table 1: Effect of the extracts from the seeds of *Trigonella foenum graecum* Linn. on isolated perfused rat uterus preparation

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Drugs / Extract</th>
<th>De Jalon solution contraction</th>
<th>Atropine (10µg/ml) + De Jalon solution contraction</th>
<th>Indomethacin (10 µg/ml) + De Jalon solution contraction</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Oxytocin (0.1 I.U.)</td>
<td>5.88 ± 0.33</td>
<td>-</td>
<td>4.51 ± 0.18 **</td>
<td>Uterine Stimulant</td>
</tr>
<tr>
<td>2.</td>
<td>Acetylcholine (1 µg/ml)</td>
<td>3.13 ± 0.17</td>
<td>1.53 ± 0.08***</td>
<td>-</td>
<td>Cholinergic activity</td>
</tr>
<tr>
<td>3.</td>
<td>TA - Total alcoholic extract (1mg/ml)</td>
<td>4.21 ± 0.20</td>
<td>4.10 ± 0.19</td>
<td>3.10 ± 0.15**</td>
<td>Uterine Stimulant</td>
</tr>
<tr>
<td>4.</td>
<td>TQ - Total aqueous extract (1mg/ml)</td>
<td>3.96 ± 0.14</td>
<td>3.86 ± 0.13</td>
<td>2.83 ± 0.08***</td>
<td>Uterine Stimulant</td>
</tr>
<tr>
<td>5.</td>
<td>PE - Petroleum ether extract (1 mg/ml)</td>
<td>3.98 ± 0.31</td>
<td>3.93 ± 0.33</td>
<td>2.70 ± 0.09**</td>
<td>Uterine Stimulant</td>
</tr>
</tbody>
</table>

Values are expressed as cm - mean ± SEM of 6 tissues. (** p < 0.01; ***p < 0.001).

Figure 2: Effect of the total alkaloidal extract and trigonelline from the seeds of *Trigonella foenum graecum* Linn. on histamine induced contractions on isolated perfused guinea pig ileum preparation. Values are expressed as mean±S.E.M. (***p<0.001)
100%. The extracts and the phytochemical of fenugreek seeds did not produce any contraction on the ileum individually. The contractile responses obtained with 1 μg of histamine were challenged against all the extracts of 1 mg/ml and trigonelline of 1 mg/ml. The histamine affinity and potency is decreased when the alkaloidal extract and trigonelline administered individually 10 min, before the administration of histamine. Thus the anti-histaminic activity was observed. Generally histamine activates H₁ receptors which leads to formation of inositol-1,4,5 triphosphate and diacyl glycerol, which turn induces the calcium mediated contraction (19). The antagonistic effect of the codes TK and TR might be due to the prevention of binding of histamine to its receptors. Hence, this experimental result may help to invent new therapeutics from this seeds for the treatment of asthma, allergy and its related diseases.

In rat colon, the spasmogenic response obtained with 1 μg of acetylcholine was considered as 100% and none of the extracts and phytochemical elicited any contractile effects. The contractile response obtained with 1 μg of acetylcholine was challenged with all the extracts and the phytochemical. It has been reported that binding of an agonist to muscarinic receptors in the smooth muscle activates G-protein which induces Ca²⁺ dependent contraction (20). The results of this study reveals that the aqueous (TQ) and glycosidal (TG) extracts when added individually to the bath containing the tissue for 10 min before the administration of acetylcholine, prevented contraction induced by acetylcholine. This indicates that the aqueous extract and glycosidal extract might contain some anti-cholinergic principles, which could have prevented the binding of acetylcholine to its receptors, through some non-specific way and blocked the contraction. This experimental result will lead a way for the use of this seeds in the treatment of diarrhoea and intestinal related diseases.

In rat uterus, the contractile response with 0.1 IU of oxytocin and 1 μg of acetylcholine was recorded on the kymograph. The codes TA, TQ and PE produced significant contraction on isolated estrogenised rat uterus preparation. This effect was not blocked by atropine (10 μg) a potent anti-cholinergic agent, but milder blockage was elicited by indomethacin (10 μg). The contractile effect of Ach was significantly blocked by atropine and the effect of oxytocin was significantly blocked by indomethacin. Normally in the sensitized uterus, the prostaglandin synthesis is increased and numbers of oxytocin receptors get increased in the endometrium, while in the myometrium, the sensitivity to the oxytocin is increased (21). In rat myometrium, as gestation progresses to term, there is a decline in muscarinic receptor-mediated phospho inositol hydrolysis, possibly because of the decrease in muscarinic receptor number to a certain extent (22). Oxytocin interacts not only with myometrial but also with endometrial receptors. It stimulates the synthesis of prostaglandins (PGF₂α) in the uterus by interacting
with endometrium. Thus prostaglandin regulates the
muscle contraction, which was significantly blocked by
indomethacin (10 μg), a potent prostaglandin
synthetase blocker. The possible mechanism of
contraction by the codes TA, TQ and PE might be due
to prostaglandin mediated contraction, because the
response was significantly blocked by indomethacin.
Further studies on this experiment will lead a way
to find out the phytochemicals present in the extracts of
Trigonella foenum graecum Linn. which possess
uterine stimulant property.
From the above study we can conclude that, the
extracts and the phytochemical of the fenugreek seeds
possess different pharmacological effects on different
types of smooth muscles. The codes TA, TQ and PE
possess uterotonic activity, code TQ and TG possess
anti-cholinergic activity and codes TK and TR possess
anti-histaminic activity. These findings will be a useful
tool for the future study in the herbal research using
fenugreek seeds.
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PHCOG MAG.: Research Article

Retardation of biofilm formation with reduced productivity of alginate as a result of Pseudomonas aeruginosa exposure to Matricaria chamomilla essential oil

Parviz Owlia¹*, Iraj Rasooli²*, Horiah Saderi³, Mousa Aliahmadi³

¹Department of Microbiology, Faculty of Medicine, Shahed University, Tehran-Iran.
²Department of Biology, Shahed University, Tehran-Iran.
³Corresponding author: No. 29, Abdollahzadeh St., Keshavarz Blvd., Department of Microbiology, Faculty of Medicine, Shahed University, Tehran-Iran.

ABSTRACT - Bacterial adhesion to solid surfaces and biofilm development are ubiquitous phenomena with several deleterious medical and economic consequences. The establishment of biofilms by alginate-producing P. aeruginosa strains is the most common mode of growth in cystic fibrosis patients with chronic lung infections, with the biofilms providing a protected environment against the host immune system and a number of antibiotics. Selected natural products that originate in plants can influence microbial biofilm formation. In this paper specific inhibition of alginate production by and antibiofilm activity of Matricaria chamomilla essential oil against Pseudomonas aeruginosa are studied. Disk diffusion method employed to evaluate P. aeruginosa inhibition exposed to the essential oil concentrations of Matricaria chamomilla L. did not show antimicrobial property. The oil composition analyzed by GC and GC/MS led to identification of 18 components of which the major ones were: guaiazulene (25.6%), (E)-8-faransens (20.1%), chamazulene (12.4%), α-bisabol oxide B (7.3%), α-bisabolol (7.3%), and hexadecanole (5.6%). Biofilm formation was studied using safranin stain employing plate reader. Alginate production was quantified in absence and in presence of the essential oil concentrations. The alginate production reduced significantly as the oil concentration increased. At the oil concentration of 0.5 μg/ml both biofilm and alginate reduced restoring alginate/biofilm ratio to normal level with decreased biofilm/alginate ratio. Use of natural agents capable of reducing biofilm formation would be useful in control of microbial pathogenicity leading to the prevention of infections of numerous biofilm producing bacterial species.

KEYWORDS: Biofilm; Alginate; Pseudomonas aeruginosa; Matricaria chamomilla L.; Essential oil

INTRODUCTION

Bacterial adhesion to solid surfaces and biofilm development are ubiquitous phenomena with several deleterious medical and economic consequences. Pseudomonas aeruginosa infections are difficult to eliminate due to their propensity to form biofilms (1) and their inherent resistance to antibiotics. The opportunistic pathogen P. aeruginosa, a ubiquitous environmental bacterium showing great adaptability and metabolic versatility (2), is the leading cause of morbidity and premature mortality in patients with cystic fibrosis (3, 4). Pseudomonas aeruginosa is remarkable in that it can cause both very acute and very chronic infections (5). Progress in understanding the pathogenesis of acute P. aeruginosa infections has implicated virulence factors including exotoxin A and type III secreted exotoxins (6, 7). The establishment of biofilms by alginate-producing P. aeruginosa strains is the most common mode of growth in cystic fibrosis patients with chronic lung infections, with the biofilms providing a protected environment against the host immune system and a number of antibiotics (8). The presence of P. aeruginosa strains with the mucoid phenotype in cystic fibrosis patients with chronic lung infections is a marker of a poor prognosis (9). Although it is not possible to eradicate P. aeruginosa, antibiotic therapy contributes to the maintenance of lung function for decades (10, 11). Therefore, the development of antibiotic resistance in P. aeruginosa is of great therapeutic concern (12). Antipseudomonal β-lactam antibiotics are widely used for the treatment of lung infections in cystic fibrosis patients. Various industries are now looking into sources of alternative,
more natural and environmentally friendly antimicrobials, antibiotics and antioxidants. Hundreds of new natural substances are being isolated and identified every year, but data concerning their biological activities are known for only some. Chamomile is one of the most widely used and well-documented medicinal plants in the world (13). It is included in the pharmacopoeia of 26 countries (14). The possibility of utilizing volatile oils is now being investigated as, although their biological activity has been known for centuries, their mode and range of action were not fully understood. Selected natural products that originate in plants can influence microbial biofilm formation. Some plant-derived compounds inhibit peptidoglycan synthesis (15), damage microbial membrane structures (16), modify bacterial membrane surface hydrophobicity (17), and modulate quorum sensing (18), all of which could influence biofilm formation. Terrestrial plants also support populations of surface-attached bacteria (19, 20) and could potentially produce phytochemicals that attenuate biofilm development through specific mechanisms. However, many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth (21-23). Owing to its glycosalyx, *Pseudomonas aeruginosa* adhesion to surfaces is facilitated while resistance to many antibiotics enables the bacterium survive easily. Chamomile contains a diverse array of secondary products including sesquiterpenes, polyacetylenes, flavonoids and coumarins. Coumarins, the group of phenypropanoid metabolites, showed antimicrobial (24) and anti-inflammatory effects (25). Successful clinical applications of *Matricaria chamomilla* have been studied (26). The present study describes specific inhibition of alginate production by and antibiofilm activity of *Matricaria chamomilla* essential oil against *Pseudomonas aeruginosa*.

**MATERIALS AND METHODS**

**Microbial strain and growth media**  
*Pseudomonas aeruginosa* 8821M was kindly supplied by Dr. Isabel Sa-corría of Instituto Superior Tecnico, Lisboa-Portugal.

**Plant and oil isolation**  
The plant origin was of Yasooj region of Iran collected during May-June 2005. The plant was identified at the department of Botany, Shahed university-Tehran as *Matricaria chamomilla* L. The shadow dried flowers were hydro distilled for 90 minutes in full glass apparatus. The oil was isolated using a Clevenger type apparatus. The extraction was carried out for 2 hours after 4-hour maceration in 500 ml of water. The oil so extracted had a specific gravity of 0.95 at 20°C, and refractive index 1.48-1.505 at 25°C was stored in dark glass bottles in a refrigerator until they were used.

**Oil analysis**  
GC analysis was performed by GC (9-A-Shimadzu) gas chromatograph equipped with a flame ionization detector. Quantitation was carried out on Euro Chrom 2000 from KNAUER by area normalization method. The analysis was carried out using a DB-5 fused-silica column (30 m×0.25mm, film thickness 0.25µm) using a temperature program of 40-250°C at a rate of 4°C/min, injector temperature 250 °C, detector temperature 265°C, carrier gas: helium (99.99%). The GC/MS unit consisted of Varian-3400 gas chromatograph coupled to a Saturn II ion trap detector. The column was same as of the GC under the same conditions stated above. The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention indices with those of authentic compounds or with literature data.

**Oil dilution**  
1 ml of oil weighing 1mg was diluted to the final volume of 2ml with DMSO (Dimethyl sulfoxide). This served as the stock solution. Mueller Hinton broth containing 0.5, 0.35 and 0.2 µg/ml of oil concentrations were made from the stock solution by adding appropriate amounts of the stock oil solution to medium.

**Antibacterial analysis**  
Unless otherwise stated, all the procedures were carried out under aseptic conditions. Disk diffusion method was employed to assess anti bacterial properties of the solvent, DMSO. Bacterial suspension equivalent to 0.5 McFarland standard units was streaked on Mueller Hinton agar plates using sterile cotton swabs. 10µl of DMSO loaded on three sterile blank disks were placed on the agar plates and were then incubated at 37°C for 24 hours. 0.2, 0.35 and 0.5 µg/ml of *Matricaria chamomilla* essential oil was added to each sterile blank disk. These concentrations were chosen on the basis of our experience and in order to avoid interference of chamomile oil colour when needed to take spectrophometric readings. The disks were placed on Mueller Hinton agar plates freshly streaked with 24 hour old bacterial suspension of *Pseudomonas aeruginosa*. The plate containing solvent on the disks served as control. Zones of inhibitions
were measured using a vernier clipper. The tests were carried out in triplicate.

**Biofilm formation and adherence assays**

The assays of biofilm formation and adherence were performed in 96-well polystyrene microplates. 190 µl of Mueller Hinton broth containing 0.5, 0.35 and 0.2 µg/ml of oil concentrations were added to each of the wells in triplicate. Bacterial cells from overnight cultures grown at 37°C were collected by centrifugation and resuspended at OD_{600}=0.01 in Mueller Hinton broth. 10 µl of the bacterial suspension was added to each well. The wells containing Mueller Hinton broth without oil, inoculated with 10 µl of the bacterial suspension served as positive control. The wells containing only Mueller Hinton broth without oil or bacteria served as negative control. The microplate was incubated at 37°C for 24 hours. The contents of the wells were drained at the end of incubation period and were then rinsed with sterile distilled deionized water (six rinses). 200 µl of 0.025% safranin solution was added to each well for 2 minutes. The safranin contents were drained and the wells were rinsed with sterile distilled deionized water (three rinses). 200 µl of ethanol:acetone (50:50 vol/vol) solution was added to each well and were allowed to stand for 15 minutes. The absorbance of the well contents, were then measured using plate reader at OD_{492}. Biofilm production was determined by using the following formula: Biofilm = (B-S)/G, where B is the amount of biofilm formed, S is the amount of safranin that adhered to the polystyrene tubes due to abiotic factors, and G is the optical density of cells grown in suspended culture (27). At least three replicate experiments were performed for each concentration of chemical that was tested.

**Quantitative assay of alginate**

500 µl of bacterial suspension corresponding to 0.5 McFarland standard solution was added to each of 50ml flasks each containing 20ml sterile Luria Bertani broth. Test flasks contained 0.2, 0.35 and 0.5 µg/ml of *Matricaria chamomilla* essential oil while without oil served as control. The flasks were then placed on an incubator shaker for 24 hours at 37°C. The samples were subjected to quantitative assay of alginate.

Alginate production was estimated as follows: 70 µl of the sample was slowly added to 600 µl of boric acid-H_{2}SO_{4} solution in a test tube placed in an ice bath. The mixture was vortexed for about 4 seconds and was placed back in the ice bath. 20 µl of 0.2% carbazole solution in ethanol was added to the test tube and was then immediately vortexed for about 4 seconds. The mixture was placed in a water bath at 55°C for 30 minutes. The absorbance was measured spectrophotometrically at 530nm. Standard alginate solutions were made by serial dilutions from the stock solution of 1000 µg/ml. Standard alginate curve was plotted using absorbance readings at 530nm (28).

**Statistical analysis**

Data obtained from the experiments were presented as mean values and the differences between control and test were analyzed using the paired t-test.

**RESULTS**

DMSO loaded disks showed no anti Pseudomonas activity on the plates, hence allowing use of this solvent as the oil diluent. 0.2, 0.35 and 0.5 µg/ml concentrations of *Matricaria chamomilla* essential oil were screened for the ability to inhibit the growth of *P. aeruginosa* 8821M. The essential oil concentrations had no effect on growth. Chemical analysis of the components of the oils led to identification of 18 components (Table 1). The major components of *Matricaria chamomilla* L. oil were guaiazulene (25.6%), (E)-B-faranesens (20.1%), chamazulene (12.4%), α-bisabol oxide B (7.3%), α-bisabolol (7.3%), and hexadecanole (5.6%). Alginate production was affected in proportionate to the increasing concentration of the oil while biofilm formation was retarded at 0.5µg/ml oil concentration (Table 2). Alginate/biofilm ratio ran in opposite directions on X axis (Fig. 1).

**DISCUSSION**

The results presented here show clearly that *Matricaria chamomilla* essential oil did not exert antimicrobial effect. *Matricaria chamomilla* essential oil has been reported to be antimicrobial against some bacteria at higher oil concentration (29). The difference in microbial susceptibility is attributable to the chemical composition of essential oil. The ineffectiveness of some oils might reflect the lack of antibacterial compounds in the plants against the microorganism under study. A possible explanation for this is that some of the plant extracts may have contained antibacterial constituents, but were not present in sufficient concentrations to be effective.
Table 1: Chemical composition of essential oil from Matricaria chamomilla L.

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>R.I.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Limonene</td>
<td>1029</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>γ-terpinene</td>
<td>1062</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>(E)-β-faranesens</td>
<td>1459</td>
<td>20.1</td>
</tr>
<tr>
<td>4</td>
<td>germacrene-D</td>
<td>1481</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>α-muurolene</td>
<td>1496</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>germacrene-A</td>
<td>1504</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>Z-γ-bisabolene</td>
<td>1515</td>
<td>2.6</td>
</tr>
<tr>
<td>8</td>
<td>Caryophyllene oxide</td>
<td>1570</td>
<td>1.2</td>
</tr>
<tr>
<td>9</td>
<td>Spathulanol</td>
<td>1578</td>
<td>1.7</td>
</tr>
<tr>
<td>10</td>
<td>α-bisabolol oxide B</td>
<td>1654</td>
<td>7.3</td>
</tr>
<tr>
<td>11</td>
<td>α-bisabolol</td>
<td>1685</td>
<td>7.3</td>
</tr>
<tr>
<td>12</td>
<td>Chamazulene</td>
<td>1729</td>
<td>12.4</td>
</tr>
<tr>
<td>13</td>
<td>α-bisabolol oxide A</td>
<td>1746</td>
<td>1.9</td>
</tr>
<tr>
<td>14</td>
<td>Guaiazulene</td>
<td>1756</td>
<td>25.6</td>
</tr>
<tr>
<td>15</td>
<td>Hexadecanole</td>
<td>1882</td>
<td>5.6</td>
</tr>
<tr>
<td>16</td>
<td>n-nonadecane</td>
<td>1891</td>
<td>1.4</td>
</tr>
<tr>
<td>17</td>
<td>Sclarene</td>
<td>1968</td>
<td>0.4</td>
</tr>
<tr>
<td>18</td>
<td>n-pentacosane</td>
<td>2500</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2: The amount (µg/ml) of alginate production and biofilm formation by P. aeruginosa exposed to various concentrations (0.2, 0.35 and 0.5 µg/ml) of M. chamomilla essential oil

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.2 µg/ml</th>
<th>0.35 µg/ml</th>
<th>0.5 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>670±27</td>
<td>549.33±13.17</td>
<td>304.5±17</td>
<td>190.33±9.9</td>
</tr>
<tr>
<td>Biofilm</td>
<td>0.66±0.12</td>
<td>0.64±0.04</td>
<td>0.63±0.05</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>Alginate/Biofilm</td>
<td>1.02</td>
<td>0.86</td>
<td>0.48</td>
<td>1.12</td>
</tr>
<tr>
<td>Biofilm/Alginate</td>
<td>0.99</td>
<td>1.17</td>
<td>2.07</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Figure 1: The amount of alginate production and biofilm formation by P. aeruginosa exposed to M. chamomilla essential oil 0.2, 0.35 and 0.5 µg/ml.
Of the major components of chamomile oil, Chamazulene, α-bisabolol and flavonoids have been reported to display antifungal properties (30). The oil under study did not have sufficient quantities of these components to impart bacteriostatic or bactericidal effect on *P. aeruginosa*. On the other hand resistance of *P. aeruginosa* to some antimicrobial agents is another factor contributing to the survival of microorganism. Endobronchial chronic infections caused by the mucoid, alginate-producing phenotype of *P. aeruginosa* is impossible to eradicate with antibiotics (31). The MICs of antimicrobial agents can be increased 100- to 1,000-fold when bacteria grow in biofilms (32). Bacterial biofilms have been defined as communities of bacteria intimately associated with each other and included within an exopolymer matrix. These biological units exhibit their own properties, which are quite different from those shown by the single species in planktonic form (33). Bacterial attachment is influenced by the surface of cells and attachment media as well as by other environmental factors (34). The results (Table 2) show that alginate production reduces significantly as the oil concentration increases. However this does not affect the biofilm formation at the oil concentrations of 0.2 and 0.35 μg/ml (Fig. 1). Understanding the pathogenesis of the chronic infections caused by *P. aeruginosa* is also progressing. Current concepts propose that biofilm formation is a key factor in chronic *Pseudomonas* airway infection in cystic fibrosis and bronchiectasis and chronic urinary tract and device-related infections (35- 38). Biofilm/alginate ratio increases significantly at the oil concentration of 0.35 μg/ml (Fig. 1) only due to the decreased level of alginate. At the oil concentration of 0.5 μg/ml (Fig. 1) both biofilm and alginate are reduced restoring alginate/biofilm ratio to the initial normal level with decreased biofilm/alginate ratio (Fig. 1). This may indicate that biofilm formation although related to, but does not merely depend upon alginate production. Biofilms confer a number of survival advantages to the bacteria, including increased resistance to antimicrobial agents (38, 39). This could be the reason as to why *P. aeruginosa* resisted *M. chamomilla* oil. However, planktonic bacteria dispersed from a biofilm usually no longer demonstrate increased levels of resistance to antibiotics, suggesting that the mode of growth is of major importance (37). The switch from the nonmucoid to the mucoid phenotype may provide profound advantages to the bacteria growing in biofilms, such as increasing their levels of resistance to antibiotics (such as tobramycin) and protecting them against the host immune system (40). Alginate appears to have important roles in the host parasite relationship. Alginate in *P. aeruginosa* may confer several selective advantages on the bacterial invader, which have been reviewed and include increased resistance to phagocytosis and reduced susceptibility to antibody-dependent bactericidal mechanisms. Alginate also provides a polyanionic barrier that may exclude cationic peptide antibiotics (41). By aggressive antibacterial chemotherapy it is possible to suppress the growth of and the damage caused by *P. aeruginosa* in the lungs of chronically infected cystic fibrosis patients. Alternatively, any treatment with natural agents that can potentially reduce biofilm formation would be useful in control of microbial pathogenicity. Consequently chronic respiratory tract infections or other infections of numerous bacterial species capable of producing biofilms might be prevented.

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REFERENCES

PHCOG MAG.: Research Article

In vitro antioxidant studies of the aerial parts of Origanum majoram Linn and Artemisia sieversiana Ehrh.


Sri Ramachandra College of Pharmacy
Sri Ramachandra Medical College and Research Institute Porur,
Chennai – 600 116.

* For Correspondence : Phone: 044 – 24768403 Ext: 8930 : E Mail: kvmanga@yahoo.com

ABSTRACT - The alcoholic extract of Origanum majoram (OM) and Artemisia sieversiana (AS) were studied for antioxidant activity on different in vitro models namely 1, 1-diphenyl, 2-picryl hydrazyl (DPPH) assay, nitric oxide assay and trichloroacetic acid based reducing power method. Ascorbic acid was also evaluated for comparison. The extracts showed dose dependent free radical scavenging property in the tested models. OM showed 96.07% inhibition of DPPH at 1000 μg and its activity at 500 μg was comparable to that of ascorbic acid at 20 μg. While the maximum percentage inhibition by OM and AS in the nitric oxide model was found to be only 41.14 and 33.63 respectively, the activity of 40 μg of OM and 10 μg of AS compares favorably with that of 20 μg ascorbic acid. AS showed marginal reductive ability. This study demonstrates the anti oxidant activity of the herbs.

KEY WORDS: Antioxidant, Origanum majoram, Artemisia sieversiana, free radicals, reductive ability.

INTRODUCTION

The role of oxygen derived free radicals in the pathogenesis of a number of degenerative disease is well known (1). Many plants contain substantial amounts of antioxidants including Vitamin C and E, carotenoids, flavonoids, tannins and thus can be utilized to scavenge the excess free radicals from the human body (2). Epidemiological studies have suggested the association between the consumption of antioxidant rich foods and beverages and the prevention of diseases (3). There is a lot of ongoing research on such plants for their potential usefulness as dietary supplements and as adjuvants for use in therapeutic management of stress related disorders.

Origanum majoram Linn. (Synonym, Majorana hortensis, Lamiaceae) is a creeping aromatic, perennial herbaceous shrub, cultivated in several states in India for use in flower garlands, bouquets and wreaths. Native of South Europe it is commonly called ‘sweet majoram’. Locally called ‘marvam’(Tamil, Hindi: marwah) it is used in traditional medicine as an emmenagogue, galactogogue and carminative(4). The essential oil from the leaves is used in hot fomentations in acute diarrhoea and is also considered an excellent external application for sprains and bruises (5). The plant possesses antibacterial, antitumour, anti inflammatory activity (6) and it reportedly inhibits platelet aggregation (7). The plant essential oil is predominantly constituted of monoterpenes and novel flavonoids have been isolated from its aerial parts (8) Considering the reported anti inflammatory activity and the flavonoid content, it is proposed to investigate its antioxidant potential.

Artemisia sieversiana Ehrh. (Synonym: Artemisia dracunculus, A.pallens, Asteraceae) is a tall perennial aromatic herb with much divided lamina extensively cultivated in mountainous districts of India. Commonly called ‘Indian wormwood’ or ‘davana’(Tamil, Hindi:‘dauna’) it is prized for its exquisite and delicate aroma with fruity fragrance. Leaf sprigs are commonly used in garlands, bouquets and religious offerings (9).

The dried flowering tops are given for inflammations (10) and urinary problems (11) in folk medicine. The plant is reportedly antimitagenic and antiviral (12). It’s essential oil is antibacterial and antifungal (13). Several aroma chemicals, flavonoids, acetylenic compounds and coumarin derivatives have been isolated from this plant (14-16). Antioxidant activity of AS is to be investigated to reason its proposed benefits in the modern context.

MATERIALS AND METHODS

All chemicals and solvents used were of analytical grade and obtained from Ranbaxy Fine Chemicals and SD Fine Chem. Ltd., Mumbai, India. Ascorbic acid was obtained from Merck Ltd., Mumbai and 1, 1-diphenyl,
2-picryl hydrazyl (DPPH) was obtained from Sigma chemicals, USA. The other chemicals used were N-(1-naphthyl) ethylene Diamine Dihydrochloride (NED), trichloro acetic acid, (TCA), Sodium nitroprusside, sulphanilamide, o- phosphoric acid, sodium chloride (NaCl), Ferrous sulphate (FeSO₄), Ferric Chloride (FeCl₃), disodium hydrogen orthophosphate, potassium dihydrogen phosphate and potassium ferricyanide.

**Plant Material**
The aerial parts of OM and AS were purchased from the local market in August 2005 and authenticated by a botanist by comparison with the preserved samples in the herbarium of our college.

**Plant extracts**
The plant parts were shade dried, powdered and extracted individually with ethanol by continuous percolation, using Soxhlet apparatus. The extracts were filtered and concentrated in vacuo and kept in a vacuum desicator for complete removal of the solvent. Alcohol extract of *Origanum majorana* (AOM) and *Artemisia sieversiana* (AAS) were obtained in the yield of 8.5% and 11.4% w/w respectively.

**In vitro antioxidant study**
AOM and AAS were tested for free radical scavenging property using DPPH and nitric oxide method. Reductive ability was tested by trichloroacetic acid method. Ascorbic acid was used as the standard antioxidant for comparision. All experiments were performed thrice and the results averaged.

**DPPH radical scavenging activity**
DPPH scavenging activity was measured by the spectrophotometric method (17). To 3 ml of an ethanolic solution of DPPH (200 μM), 0.05 ml of test extracts/ascorbic acid (20 μg) dissolved in ethanol were added. Test extracts were prepared in different concentrations (4-1000μg). The solutions were incubated at 37° for 30 min, absorbance measured at 517 nm using Systronics 118 model spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with the extract) using the formula (18)

\[
\text{Percentage inhibition} = \frac{(\text{Absorbance of control - Absorbance of test}) \times 100}{\text{Absorbance of control}}
\]

**Nitric oxide scavenging activity** (19) - Nitric oxide radicals were generated from sodium nitroprusside solution at physiological pH. 1 ml of sodium nitroprusside (10mm) was mixed with 1 ml of the test extracts / ascorbic acid (20 μg) in phosphate buffer (pH 7.4). The test extracts were prepared in different concentrations (10-1000 μg). The mixture was incubated at 25° for 150 mins. To 1.5 ml of the incubated solution, 1 ml of Griess’ reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added. Absorbance was read at 546 nm and percentage inhibition calculated.

**Reductive ability (20)**
Reducing power of the test extracts was determined based on the ability of antioxidants to form coloured complex with potassium ferricyanide, TCA and FeCl₃. 1 ml of the test extracts (100-800 μg) / ascorbic acid (20 μg) in ethanol were mixed with 2.5 ml potassium ferricyanide (1%) and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50° C for 20 min. 2.5 ml TCA (10%) were added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml water and 0.5 ml FeCl₃ (0.1%). Absorbance was measured at 700 nm.

**Statistical analysis**
Linear regression analysis was used to calculate IC₅₀ values wherever needed.

**RESULTS**
AOM and AAS in graded concentrations were tested for their antioxidant activity in three different *in vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in the models studied. The maximum percentage inhibition of DPPH by AOM and AAS was 96.07 and 64.48% respectively at 1 mg concentration (Table 1). Standard drug ascorbic acid showed 93.58% inhibition of the DPPH radical at 20 μg. IC₅₀ value of AOM and AAS was respectively 65 μg and 625 μg.

In the nitric oxide model, the maximum percentage of inhibition of nitric oxide radicals of AOM and AAS was 41.14% and 33.63% respectively (Table 2). However, ascorbic acid at 20 μg caused only 7.51% inhibition which is similar to the inhibition of 40 μg of AOM and 10 μg of AAS.

The reducing power of AOM and AAS was also dose dependent and shown in Table 3. The maximum absorbance of AOM at 800 μg, compares favourably with ascorbic acid. On a comparative basis AOM was better at quenching DPPH and nitric oxide radicals than AAS. Also its reductive ability is far greater than AAS.

**DISCUSSION**
DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule.
Table 1 - DPPH scavenging activity of AOM and AAS

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOM</td>
</tr>
<tr>
<td>1000</td>
<td>96.07 ± 0.052</td>
</tr>
<tr>
<td>500</td>
<td>93.45 ± 0.084</td>
</tr>
<tr>
<td>250</td>
<td>89.52 ± 0.073</td>
</tr>
<tr>
<td>125</td>
<td>84.14 ± 0.068</td>
</tr>
<tr>
<td>62</td>
<td>47.84 ± 0.044</td>
</tr>
<tr>
<td>32</td>
<td>15.72 ± 0.011</td>
</tr>
<tr>
<td>16</td>
<td>3.67 ± 0.015</td>
</tr>
<tr>
<td>10</td>
<td>3.01 ± 0.032</td>
</tr>
<tr>
<td>7</td>
<td>0.65 ± 0.084</td>
</tr>
<tr>
<td>5</td>
<td>0.39 ± 0.038</td>
</tr>
</tbody>
</table>

% inhibition of DPPH due to ascorbic acid (20µg) is 93.58.
Values are mean of triplicate determinations

Table 2 - Nitric oxide scavenging activity of AOM and AA

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOM</td>
</tr>
<tr>
<td>1000</td>
<td>41.14 ± 0.058</td>
</tr>
<tr>
<td>800</td>
<td>33.02 ± 0.008</td>
</tr>
<tr>
<td>600</td>
<td>30.04 ± 0.015</td>
</tr>
<tr>
<td>400</td>
<td>28.36 ± 0.004</td>
</tr>
<tr>
<td>200</td>
<td>23.54 ± 0.016</td>
</tr>
<tr>
<td>100</td>
<td>10.76 ± 0.084</td>
</tr>
<tr>
<td>40</td>
<td>6.95 ± 0.064</td>
</tr>
<tr>
<td>20</td>
<td>5.83 ± 0.042</td>
</tr>
<tr>
<td>10</td>
<td>3.92 ± 0.016</td>
</tr>
</tbody>
</table>

% inhibition of NO due to ascorbic acid (20µg) is 7.51.
Values are mean of triplicate determinations

Table 3 - Reductive ability of AOM and AAS

<table>
<thead>
<tr>
<th>Concentration (µg)</th>
<th>Absorbance at AOM</th>
<th>700 nm. AAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>0.588 ± 0.060</td>
<td>0.126 ± 0.032</td>
</tr>
<tr>
<td>600</td>
<td>0.414 ± 0.068</td>
<td>0.092 ± 0.054</td>
</tr>
<tr>
<td>400</td>
<td>0.412 ± 0.042</td>
<td>0.03 ± 0.044</td>
</tr>
<tr>
<td>200</td>
<td>0.0158 ± 0.021</td>
<td>0.025 ± 0.012</td>
</tr>
<tr>
<td>100</td>
<td>0.027 ± 0.007</td>
<td>0.003 ± 0.001</td>
</tr>
</tbody>
</table>

Absorbance due to ascorbic acid (20µg) is 0.605.
Values are mean ± SEM of triplicate determinations

DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colour stoichiometrically depending on the number of electrons taken up(21). From the results it may be postulated that both the plant extracts have hydrogen donors thus scavenging the free radical DPPH.

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, alzheimer’s, and arthritis (22). Oxygen reacts with the excess NO to generate nitrite and peroxynitrite anions, which act as free radicals. In the present study the nitrite produced by
the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by AOM and AAS. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. It is to be noted that AOM and AAS have caused a greater inhibition than ascorbic acid which has shown only 7.51% inhibition of NO. It is known that ascorbic acid acts as a pro oxidant in vitro in the presence of transition metal ions such as iron (23). This could explain its meagre antioxidant effect in this method. This effect is however unlikely to be important in vivo where metal ions are sequestered and other reductants are present. An increase in absorbance in the reducing power method implies that extracts are capable of donating hydrogen atoms in a dose dependent manner.

It is evident from the results that AOM has a higher antioxidant potential than AAS. Several flavonoids apart from chlorogenic acid have been reported from OM. These could be the antioxidant principles mediating the anti inflammatory activity reported earlier for this herb. The results thus support the folklore claim of the usefulness of the herb in inflammatory conditions.

The radical scavenging potential of AS is explained by the presence of flavonoids, acetylenic compounds and ascorbic acid in the herb. It also correlates with the anti mutagenic activity reported earlier, as chemicals that scavenge free radicals prevent DNA strand breaks (24).

Demonstration of the antioxidant potential of the herbs, especially in view of the presence of a rich spectrum of bio active molecules of therapeutic significance, makes them likely candidates for bio activity guided fractionation of useful phytomolecules.

REFERENCES


PHCOG MAG.: Research Article

Hepatoprotective activity of *Bacopa monnieri* L. against ethanol - induced hepatotoxicity in rats

T. Ghosh\(^a\), T. K. Maity\(^b\), M. Das\(^a\), A. Bose\(^a\), G. K. Dash\(^a\)

\(^a\)Institute of Pharmacy and Technology, Salipur, Cuttack-754202, Orissa, India
\(^b\)Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India

Corresponding author: E-mail address: tghosh75@yahoo.co.in

**ABSTRACT** - This study was designed to test the hypothesis that ethanolic extract of *Bacopa monnieri* aerial parts (EBM) protects against ethanol - induced liver injury in rats. Crude ethanolic extract of *B. monnieri* was investigated for hepatoprotective activity in albino rats at 300 mg/kg, orally and compared with standard drug Silymarin (25 mg/kg, PO). Results show that EBM was effective in blunting ethanol-induced enhanced activities of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), level of serum bilirubin (both total and direct), serum total cholesterol, liver weight loss and was also effective in reducing ethanol-induced lipid peroxidation both in *vitro* and *in vivo*. EBM on the other hand was found to enhance level of HDL cholesterol in ethanol-induced hepatotoxic rats. Furthermore, EBM could also blunt ethanol-induced suppressed activities of superoxide dismutase (SOD), catalase (CAT) and decreased level of reduced glutathione (GSH). Results of hepatocellular damage caused by ethanol and its recovery by EBM, suggest that it might be considered as a potential source of natural hepatoprotective agent, which could be related to the free radical scavenging properties of saponins present in high concentration in the extract.

**KEY WORDS:** *Bacopa monnieri* L.; Hepatoprotective activity; Antioxidants; Saponins.

**INTRODUCTION**

Alcohol dependency is a major health and socio-economic problem throughout the world (1, 2). It has been observed that almost all ingested alcohol is metabolized in the liver and excessive alcohol use can lead to acute and chronic liver disease (3). It has further been observed that most of the consumed alcohol is eventually broken down by the liver and the products generated and accumulated during alcohol metabolism (e.g. acetaldehyde) are more toxic than alcohol itself (3). In addition, a group of metabolic products called free radicals can damage liver cells and promote inflammation, impairing vital functions such as energy production. The body’s natural defenses against free radicals (e.g. antioxidants) are inhibited by alcohol consumption, leading to increased liver damage (3). Despite great progress made in the field in the past two decades, development of suitable medications for the treatment of alcohol dependency or alcohol-induced health injury remains a challenging goal for alcohol research.

*Bacopa monnieri* L. (Scrophulariaceae) is a creeping, glabrous, succulent herb, rooting at nodes, distributed throughout India in all plain districts, ascending to an altitude of 1,320 m (4). The plant is reported to contain tetracyclic triterpenoid saponins, bacosides A, B, C, hersaponin, alkaloids viz. herpestine and brahmine and flavonoids (4-6). Saponins are natural products, which have been shown to possess antioxidant property (7-9). Studies have confirmed that indeed oxidative stress plays an important role in the initiation and progression of alcohol liver disease (ALD) (10-12). As *B. monnieri* contains large amounts of saponins it was thought worthwhile to investigate the hepatoprotective activity of the aerial parts of *Bacopa monnieri* Linn. in a scientific manner.

**MATERIALS AND METHODS**

**Plant material**

The plant was identified by the taxonomists of the Botanical Survey of India, Govt. of India, Shibpur, Howrah. After authentication, fresh aerial parts of the young and matured plants were collected in bulk from the rural belt of Salipur, Orissa, India during early summer, washed, shade dried and then milled in to coarse powder by a mechanical grinder. The powdered plant material (400 g) was defatted with petroleum ether (60-80°C) and then extracted with 1.5 litre of ethanol (95%) in a soxhlet apparatus (13). The solvent was removed under reduced pressure, which obtained a greenish-black sticky residue (yield: 11.6% w/w with...
respect to dried plant material). The dried extract was stored in a desiccator till further study.

**Animals**

Male Wistar rats weighing 120 ±5 g were used in the experiment. They were maintained in a 12 h light/dark cycle at 25 ±2°C. They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC).

**Diet and treatment**

Animals were divided into four groups: Group I (control), Group II (ethanol treated), Group III (ethanol + EBM), Group IV (ethanol + silymarin). Animals of groups II, III and IV were fed with 15% (v/v) ethanol (14) at a single dose per day for 30 days by oral route. Simultaneously, but at different hours of the day, animals of groups III and group IV were fed with 300 mg/kg EBM orally and 25 mg/kg silymarin orally for 30 days at a single dose of 1 ml/100 g body wt/day respectively. Animals of group I was administered orally deionized water, 1 ml/100 g body wt/day, as vehicle. Daily records of body weight of all groups of animals were maintained during the whole experimental period.

**Acute toxicity study**

The test was carried out as suggested by Seth et al., 1972 (15). Swiss albino mice of either sex weighing between 25 - 30 g were divided into nine groups of six animals in each. The control group received normal saline (2 ml/kg, PO). The other groups received 100, 200, 300, 600, 800, 1000, 2000, 3000 mg/kg of the test extract, respectively. Immediately after dosing, the animals were observed continuously for the first 4 hours for any behavioral changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any. The observations were made twice daily, one at 7 a.m. and another at 7 p.m.

**Serum analysis**

After the treatment period, the animals of all groups were anaesthetized and sacrificed. Blood was drawn from heart and serum was separated for the assay of serum glutamate oxaloacetate transaminase (SGOT) (16), serum glutamate pyruvate transaminase (SGPT) (16), alkaline phosphatase (ALP) (17), bilirubin (direct and total) (18) and cholesterol (total and HDL) (19) using analytical kits from Span Diagnostics Ltd., Surat, India.

**In vitro antioxidant activity**

The inhibitory effect of EBM on ethanol-induced lipid peroxidation in mice liver homogenate was determined using TBA- MDA adduct according to the modified method of Yuda et al., 1999 (20). A mixture containing 0.5 ml of normal liver homogenate, 0.1 ml of Tris- HCl buffer (pH 7.2), 0.1 ml of ethanol and 0.05 ml of various concentrations of EBM (0.01, 0.1 and 1.0 mg/ml) were incubated for 1hr at 37°C. After incubation 9 ml of distilled water and 2 ml of 0.6% Thiobarbituric acid (TBA) were added to 0.5 ml of incubated solution and shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling, 5 ml of n- butanol was added and the mixture was again shaken vigorously. The n- butanol layer was separated by centrifugation at 1000 g for 30 min and the absorbance was measured at 532 nm (Wong et. al., 1987) (21).

**In vivo antioxidant activity**

After the treatment period following study, the animals were deprived of food overnight and sacrificed by cervical dislocation. The livers were dissected out, washed in ice-cold saline, patted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation (LPO) by the method of Fraga et al., 1988 (22). A part of homogenate after precipitating proteins with trichloro acetic acid (TCA) was used for estimation of reduced glutathione (GSH) by the method of Ellman et al., 1959 (23). The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of superoxide dismutase (SOD) by the method described by Kakkar et al., 1984 (24) and catalase (CAT) activity was measured by the method of Maehly et al., 1954 (25). Protein estimation was done as per the method of Lowry et. al., 1951 (26).

**Statistical analysis**

Statistical significance was determined by one way analysis of variance (ANOVA) followed by Dunnet’s t-test (*P* < 0.05) to compare group means.

**RESULTS**

**Serum analysis**

In acute toxicity study, it was found that the extract induced sedation and temporary postural defect at all tested doses. However, there was no mortality at any of the tested doses till the end of 14 days of observation. Rats subjected to ethanol only, developed significant (*P*<0.05) hepatocellular damage as evident from significant increase in serum activities of GOT, GPT, ALP and bilirubin concentration as compared to
Table 1: Effect of EBM (300 mg/kg p.o.) on SGOT, SGPT, ALP, bilirubin (total and direct), cholesterol (total and HDL) and liver weight in ethanol-induced hepatotoxic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>SGOT (U/ml)</th>
<th>SGPT (U/ml)</th>
<th>ALP (KA units)</th>
<th>Bilirubin (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>46.33 ± 0.95</td>
<td>55.33 ± 0.67</td>
<td>78.00 ± 1.79</td>
<td>0.57 ± 0.08</td>
<td>116.42 ± 1.90</td>
<td>9.98 ± 0.61</td>
</tr>
<tr>
<td>II</td>
<td>Ethanol treated</td>
<td>135.17 ± 3.11</td>
<td>117.33 ± 3.98</td>
<td>159.33 ± 4.53</td>
<td>6.05 ± 0.73</td>
<td>184.78 ± 1.51</td>
<td>10.90 ± 8.53</td>
</tr>
<tr>
<td>III</td>
<td>Ethanol + Extract</td>
<td>62.33 ± 3.62</td>
<td>64.50 ± 2.88</td>
<td>112.83 ± 4.49</td>
<td>0.95 ± 0.21</td>
<td>136.77 ± 10.17</td>
<td>11.17 ± 10.75</td>
</tr>
<tr>
<td>IV</td>
<td>Ethanol + Silymarin</td>
<td>53.50 ± 2.95</td>
<td>62.00 ± 2.92</td>
<td>91.33 ± 5.06</td>
<td>0.96 ± 0.34</td>
<td>143.7 ± 20.18</td>
<td>10.50 ± 10.50</td>
</tr>
</tbody>
</table>

All values are Mean ± SEM, n=6 rats in each group. *P < 0.05 as compared with Group I. **P < 0.05 as compared with Group II.

Table 2: Effect of EBM on ethanol-induced in vitro lipid peroxidation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>% inhibition</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic extract</td>
<td>50</td>
<td>22.66 ± 1.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>32.45 ± 2.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>47.86 ± 2.37</td>
<td>216.59</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>57.59 ± 1.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>62.22 ± 2.41</td>
<td></td>
</tr>
<tr>
<td>α-tocopherol</td>
<td></td>
<td></td>
<td>190.22</td>
</tr>
</tbody>
</table>

n=3, Values are Mean ± S.E.M.
**Table 3: Effect of EBM on LPO, antioxidant enzymes and GSH in liver of ethanol-induced hepatotoxic rats in vivo**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>LPO(a)</th>
<th>SOD(b)</th>
<th>CAT(c)</th>
<th>GSH(d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control</td>
<td>1.69 ± 0.27</td>
<td>12.59 ± 0.42</td>
<td>64.40 ± 3.25</td>
<td>61.59 ± 2.11</td>
</tr>
<tr>
<td>II</td>
<td>Ethanol treated</td>
<td>7.32 ± 0.23</td>
<td>3.66 ± 0.17</td>
<td>39.80 ± 2.13(^*)</td>
<td>34.84 ± 1.44(^*)</td>
</tr>
<tr>
<td>III</td>
<td>Ethanol + Extract</td>
<td>3.79 ± 0.33(^*)</td>
<td>8.74 ± 0.26(^*)</td>
<td>54.52 ± 3.71(^**)</td>
<td>58.00 ± 1.56(^**)</td>
</tr>
<tr>
<td>IV</td>
<td>Ethanol + Silymarin</td>
<td>3.23 ± 0.15(^*)</td>
<td>9.49 ± 0.40(^*)</td>
<td>56.64 ± 2.80(^**)</td>
<td>54.68 ± 1.88(^**)</td>
</tr>
</tbody>
</table>

All values are Mean ± SEM, \(n=6\) rats in each group. \(^*\)\(P < 0.05\) as compared with Group I. \(^**\)\(P < 0.05\) as compared with Group II. \(a=\) nmole of MDA/mg of protein. \(b=\) Units/mg of protein. \(c=\) µmole of H\(_2\)O\(_2\) consumed/min/mg of protein. \(d=\) µg/mg of protein.

Normal control group, which has been used as reliable markers of hepatotoxicity (Table 1). Oral administration of EBM (300 mg/kg) exhibited significant reduction \((P<0.05)\) in ethanol-induced increase in levels of GOT, GPT, ALP and bilirubin concentration. Treatment with silymarin also reversed the hepatotoxicity significantly \((P<0.05)\). Table 1 also revealed that total cholesterol level of serum of rats treated only with ethanol increased significantly \((p<0.05)\) while HDL level decreased significantly \((p<0.05)\) with respect to control group. But, EBM was successful in blunting this ethanol-induced increase in serum cholesterol level and decrease in HDL level, which is comparable with the reference drug silymarin.

**Liver weight**
Liver weight of rats treated with ethanol only decreased significantly \((p<0.05)\), which is prevented by EBM and silymarin (Table 1).

**In vitro lipid peroxidation**
Ethanol-induced in vitro lipid peroxidation study revealed that EBM has significant anti lipid peroxidation potential with IC\(_{50}\) value being 216.59 µg/ml, which is comparable with the reference drug α-tocopherol (Table 2).

**In vivo antioxidant activity**
In vivo lipid peroxidation study revealed that ethanol treated group showed significant increase \((p<0.05)\) in malondialdehyde (MDA) level when compared with normal control group. EBM and silymarin were able to significantly prevent \((p<0.05)\) this rise in MDA level (Table 3).

There was a marked decrease in the level of GSH and the activities of SOD and CAT in ethanol treated group when compared with normal control group. The GSH level and activities of SOD and CAT were significantly increased \((p<0.05)\) in EBM and silymarin treated groups (Table 3).

**DISCUSSION**
It has been found that EBM effectively could prevent ethanol-induced biochemical changes of liver toxicity. The hepatoprotective activity of the ethanolic extract was monitored by estimating serum transaminases, serum alkaline phosphatase and bilirubin, which give a good idea about the functional state of the liver (27). The increase in the levels of serum bilirubin reflected the level of jaundice and increase of transaminases and ALP was the clear indications of cellular leakage and loss of functional integrity of cell membrane (28). There was a significant decrease in mean liver weight of the animals in ethanol-induced group, which could be blunted significantly by EBM. This decrease in liver weight by ethanol-induced hepatotoxic rats and its recovery by EBM, suggests that EBM possibly has a positive anabolic effect.

Formation of ROS, oxidative stress and hepatocellular injury have been implicated to alcoholic liver disease. It has been documented that Kupffer cells are the major sources of ROS during chronic ethanol consumption, and these are primed and activated for enhanced formation of pro-inflammatory factors (11). Additionally, alcohol-induced liver injury has been associated with increased amount of lipid peroxidation (29). Indeed, EBM supplementation in our study was potentially effective in blunting lipid peroxidation, suggesting that EBM possibly has antioxidant property to reduce ethanol-induced membrane lipid peroxidation and thereby to preserve membrane structure. It may thus be plausible that in our study, loss of membrane structure and integrity because of lipid peroxidation was accompanied with an elevated level of activities of SGOT, SGPT, ALP and bilirubin.
Our study further revealed that chronic exposure to ethanol decreased the activities of the ROS scavenging enzymes, viz. SOD and CAT. This is in line with assumption suggested earlier by Sandhir and Gill (1), that decrease in the activity of antioxidant enzymes SOD, CAT and GSH following ethanol exposure may be due to the damaging effects of free radicals, or alternatively could be due to a direct effect of acetaldehyde, formed from oxidation of ethanol, on these enzymes. In our studies, it reveals that EBM could restore the activity of both these antioxidant enzymes and possibly could reduce generation of free radicals and hepatocelellular damage.

GSH is a naturally occurring antioxidant important in the antioxidant defence of the body. It has been reported that determination of GSH, can serve as a key to know the amount of antioxidant reserve in the blood and probably in the organism and also, contribute in evaluating the possibilities available for the recuperation of alcoholic patients (30, 31). Therefore, the levels of glutathione are of critical importance in liver injury caused by toxic substances such as ethanol.

It has been claimed that binding of acetaldehyde, a metabolite of ethanol, with GSH may contribute to reduction in the levels of GSH (1). Our results are in line with this earlier report because we found that after EBM-supplementation, elevated GSH level in rats with ethanol could be blunted to normal level. This ability of EBM to protect the liver from ethanol-induced damage might be attributed to its ability to restore the activity of antioxidative enzymes. Thus, results of these studies together with those of earlier ones, suggest that EBM has an ability to protect the liver from ethanol-induced damage through its direct antioxidative effect.

In summary, we demonstrate that EBM prevents ethanol-induced oxidative stress and hepatic injury. Since these models of hepatic damage in the rat simulate many of the features of human liver pathology, we suggest that natural antioxidants and scavenging agents in EBM might be effective as plant hepatoprotectors and thus may have some obvious therapeutic implications. Therefore, it seems logical to infer that EBM, because of its antioxidant property, might be capable of protecting the hepatic tissue from ethanol-induced liver injury. As B. monnieri contains large amounts of saponins it might be suspected that the hepatoprotective activity might be due to the presence of saponins in the extract. The activity may be attributed to their protective action on lipid peroxidation and at the same time the enhancing effects on cellular antioxidant defense contributing to the protection against oxidative damage in ethanol-induced hepatotoxicity. Further studies regarding the isolation and characterisation of the active principles responsible for hepatoprotective activity is currently under progress.

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The authors are thankful to the management of Institute of Pharmacy and Technology, Salipur, India and the authorities of Jadavpur University, Kolkata, India for providing necessary facilities to carry out the research work. The authors are also thankful to the taxonomists of Botanical Survey of India, Shibpur, Howrah, India for proper identification of the plant.

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PHCOG MAG.: Research Article

Hypoglycemic and Anti-hyperglycemic Effect of Alcoholic Extract of Benincasa hispida in Normal and in Alloxan Induced Diabetic Rats

G. R. Battu\textsuperscript{a}, S. N. Mamidipalli\textsuperscript{a}, R.Parimi\textsuperscript{a}, R.K.Viriyala\textsuperscript{a}, R.P.Patchula\textsuperscript{a}, L.R.Mood\textsuperscript{a}

\textsuperscript{a}Pharmacognosy and Phytochemistry Division, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam- 530 003.

Correspondence: sanjithmamidipalli@yahoo.co.in

ABSTRACT - The hypoglycemic and anti-hyperglycemic effect of alcoholic extract of Benincasa hispida was investigated in normal and alloxan induced diabetic rats. A single oral administration of alcoholic extract of Benincasa hispida at doses 50, 100, 200mg/kg produced a significant blood glucose reduction in a dose dependent manner. A bioguided extraction and fractionation of alcoholic extract of the stem of Benincasa hispida afforded three compounds, B-sitosterol, \(\alpha\)-amyrin, quercetin. The alcoholic extract at 200mg/kg significantly reduced the blood glucose levels and equipotent with that of the standard drug Tolbutamide.

KEY WORDS: Diabetes mellitus, Benincasa hispida, Alloxan.

INTRODUCTION

Diabetes mellitus is a group of syndromes characterized by hypoglycaemia, altered metabolism of lipids, carbohydrates and proteins, it is an increased risk of complications from vascular diseases (1). Chronic hyperglycemia during diabetes causes glycation of body proteins that in turn leads to secondary complications effecting eyes, kidneys, nerves and arteries (2). These may be delayed, decreased or prevented by maintaining blood glucose values close to normal. The increasing number of aging population, consumption of calories rich diet, obesity and sedentary life style have lead to tremendous increase in the number of diabetics worldwide. According to W.H.O projections, the prevalence of diabetes is likely to increase by 35%. Currently there are over 150 million diabetics worldwide and this is likely to increase to 300 million or more by the year 2025. Statistical projections about India suggest that the number of diabetics will rise from 15 million in 1995 to 57 million in the year 2025 making it the country with the highest number of diabetics in the world (3,4). It is apparent that due to the side effects of the currently used drugs, there is a need for safe agents with minimal adverse effects, which can be taken for long duration. Recently, the search for appropriate hypoglycemic agents has been focused on plants used in traditional medicine partly because of leads provided by traditional medicine to natural products that may be better treatments than currently used drugs (5). India is a country with a vast reserve of natural resources and rich history of traditional medicine (6). In the indigenous system of medicine (Ayurveda), a mention was made on many number of plants for the cure of diabetes or ‘madhumeha’ and some of them have been experimentally evaluated and the active principles were isolated (7, 8, 9, 10, 11). How ever, search for novel anti diabetic drugs continues.

The family contains 119 genera and contains around 825 species. Plants belonging to the Benincasa species have been the subjects of many investigations for their biologically active components. Some species of Benincasa have been used as medicinal plants for the treatment of diabetes, urinary infection, summer fever, (12) epilepsy, insanity and other nervous diseases, gonorrhoea, demulcent facilitates pus drainage.(13,14).The rind of the fruit is used as diuretic(15).

MATERIALS AND METHODS

Plant

The stem of the plant Benincasa hispida was collected from a place called Gujarathi peta, Sriakakulam dist. and A.P, India in August-2001. It was confirmed by Associate professor, Dr.M.Venkaiah (Department of Botany, A.U, Vishakapatnam). A voucher specimen (BGR-2006) is deposited in herbarium of Andhra University, Visakhapatnam.
**Animals**

Laboratory breed Sprague Dawley rats of either sex weighing 200-225 g were employed for the study. All animals were procured from National Institute of Nutrition, Hyderabad. The rats were maintained under standard laboratory conditions at 25±2°C, relative humidity 50±15% and normal photo period (12hr dark /12hr light). Commercial pellet diet (Rattan Brothers, India) and water were provided adlibitum. The experimental protocol has been approved by the Institutional Animal Ethics Committee and by the regulatory body of the government (Reg No-516/01/A/CPCSEA).

**Drugs**

Alloxan mono hydrate was purchased from Sigma Chemicals (St Louis,U.S.A). All other chemicals used for this study were of analytical grade.

**Extraction and Isolation Procedure**

Freshly collected plant material stem was cut into small pieces and shade dried. The dried stems were powdered in Willey mill. The powdered stem (700g) was extracted with methanol (4L) by process of continuous extraction (soxhlation). The crude extract was evaporated to dryness in a rotary film evaporator. 1 g of alcoholic extract equivalent to 30.33 g of crude drug was obtained. β-sitosterol was crystallized from hexane as colourless fine needles with m.p136 to 138°C, α-amyrin was obtained from the fractions of hexane:ethylacetate(90:10) of colum chromatography from methanol as colourless needles with m.p 186 to 187°C, β-sitosterol-3-o-β-D-glucoside was obtained from fractions of 51-60 of colum chromatography of ethylacetate extract and crystallized from acetone as white shining crystalline needles with m.p 289-292°C, Quercetin was soluble in ether acetone ethylacetate and methanol. The compound was crystallized twice from methanol as yellow needles with m.p 310°C. All the above compounds were confirmed by melting point and Co-TLC studies with the authentic sample.

**Toxicity Evaluation in Mice**

The alcoholic extract was tested for its acute and short-term toxicity (if any) in mice. To determine acute toxicity, a single oral administration of the alcoholic extract at doses of 0.25,0.5,0.75,1.0 g/kg were administered to different groups of mice (2 mice were used for each group, control mice received 1%SodiumCMC) mortality and behavior of the animals were observed periodically for 48hr. The animals were observed continuously from the initial at the 4th, 6th, 24th, 48th hours following drug administration. The parameters observed were grooming hyperactivity, sedation, loss righting, respiratory rate and convulsion. To study short-term toxicity, 3 groups of mice each containing 6 male mice (20-25g body weight) were used. Group1 was kept as control and Group2,Group3 received 200and 400mg/kg alcoholic extract respectively in 1%SodiumCMC. The drug was administered daily for 14 days (p.o) control group received 1%SodiumCMC in an identical manner. The behaviors of the animals were observed daily for 1hr in the forenoon (10to 11am) for 14days. Initial and final body weights, water and food intake, state of tool and body temperature was observed.

**Induction of Diabetes**

Animals were allowed to fast 18hr and were injected with alloxan monohydrate dissolved in sterile normal saline at a dose of 140mg/kg body weight intraperitoneally. After 2 weeks, diabetic rats (250-350mg/dl) were used for the experiment.

**Experimental Design**

In the experiment a total number of 60 (30 normal and 30 diabetic) surviving rats were used. The rats were divided into 10 groups, each group consisting of 6 animals. Group1 Normal rats treated with vehicle (1% sodium CMC) and served as normal control, Group2 Normal rats treated with alcoholic extract of *B.hispidá* at dose of 50 mg/kg, Group3 Normal rats treated with alcoholic extract of *B.hispidá* at dose of 100 mg/kg, Group 4 Normal rats treated with alcoholic extract of *B.hispidá* at dose of 200 mg/kg, Group 5 treated with Tolbutamide 40mg/kg. Group6 Diabetic rats treated with vehicle (1% sodium CMC) served as diabetic control. Group7 Diabetic rats were treated with alcoholic extract of *B.hispidá* at doses of 50mg/kg, Group8 Diabetic rats were treated with alcoholic extract of *B.hispidá* at doses of 100mg/kg, Group9 Diabetic rats were treated with alcoholic extract of *B.hispidá* at doses of 200mg/kg and Group 10 treated with Tolbutamide 40mg/kg. All the doses were administered orally.

**Estimation of Blood Glucose**

The rats were fasted for 18hr and blood samples were collected by puncture of retro-orbital plexus immediately with capillary tube under ether anesthesia into glass vials containing a small quantity of a mixture of potassium oxalate and sodium fluoride as an anticoagulant at 0hr (before treatment) and 2, 4, 6, 8, 12, 24hr (after treatment). The plasma blood glucose levels were determined by using GOD-POD method (16).
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**Statistical Analysis**

All values were expressed as Mean±S.E.M. The differences were compared using one way analysis of variance (ANOVA) followed by Dunnett’s t test. p values <0.05 were considered as significant.

**RESULTS**

The effect of different doses of alcoholic extract of *Benincasa hispida* on fasting blood sugar levels in normal rats were assessed at different time intervals. The maximum percentage blood glucose reduction with 50, 100 and 200mg /kg doses of *B.hispida* at 6hr were 21.32%, 45.87% and 66.06% respectively. Tolbutamide 40mg/kg dose produced 64.89% blood glucose reduction in normal rats and results were shown in table 1. The anti hyperglycemic effect of different doses of alcoholic extract of *B.hispida* on fasting blood glucose levels in diabetic rats were assessed at different time intervals. The percentage blood glucose reduction with 50, 100 and 200mg /kg dose of *B. hispida* at 6hr were 52.3%, 54.25% and 61.9% respectively. Tolbutamide (40mg/kg) produced 64.89% blood glucose reductions in alloxan induced diabetic rats were shown in table 2.

**DISCUSSION**

The present study was conducted to evaluate the hypoglycemic and anti hyperglycemic activity of *Benincasa hispida* which is a very new herbal drug that was firstly identified by us to get a berth in the group of antidiabetic herbal drugs. In this study, the alcoholic extract of *Benincasa hispida* produced a dose dependent percentage blood glucose reduction in normal and diabetic group. In normal treated groups a significant percentage blood glucose reduction was observed up to 24hr and maximum percentage blood glucose reduction was observed at 6hr, where as in diabetic groups also significant reduction in blood glucose was maintained up to 24hr and maximum at 6hr. The percentage blood glucose reduction produced by the extract at 200mg/kg in diabetic groups is highly significant (p<0.001), greater than the percentage reduction observed in Tolbutamide (standard) treated groups.

**Phytochemical analysis of Benincasa hispida** alcoholic extract shows the presence of alkaloids, flavonoids, saponins and steroids. Different mechanisms of action to reduce blood glucose levels with the help of plant extracts already exist. Some plants exhibit properties similar to the well-known sulfonylurea drugs like Tolbutamide; they reduce blood glucose in normoglycemic animals (17, 18). Flavonoids, sterols/terpenoids, alkaloids and phenolics are known to be bioactive anti diabetic principles (9,10,19,20). Flavonoids are known to regenerate the damaged beta cells in the alloxan diabetic rats (21). Alloxan induces diabetes by destroying β-cells (22). In the normoglycemic diabetic rats, the alcoholic extract was effective, which suggests the presence of orally active insulin like compound as reported for *Optunia* species (23). Increased peripheral utilization and inhibition of the proximal tubular reabsorption mechanism for glucose in the kidney, if any, can also contribute to a glucose lowering effect (24).

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**Table 1. Percentage blood glucose reduction in normal fasting rats after treatment with Benincasa hispida**

<table>
<thead>
<tr>
<th>Hours</th>
<th>Control</th>
<th>Standard (Tolbutamide)</th>
<th>B. hispida (50mg/kg)</th>
<th>B. hispida (100mg/kg)</th>
<th>B. hispida (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>1</td>
<td>1.115±0.644</td>
<td>6.488±3.835</td>
<td>19.830±10.333</td>
<td>5.113±0.973</td>
<td>17.285±7.995</td>
</tr>
<tr>
<td>2</td>
<td>2.815±0.731</td>
<td>1.90±4.838</td>
<td>15.010±7.536</td>
<td>12.1±4.742</td>
<td>43.743±2.417</td>
</tr>
<tr>
<td>3</td>
<td>3.945±0.303</td>
<td>28.25±7.498</td>
<td>17.610±10.027</td>
<td>25.337±4.677</td>
<td>30.672±2.081</td>
</tr>
<tr>
<td>4</td>
<td>5.932±0.854</td>
<td>44.21±5.971</td>
<td>17.325±8.345</td>
<td>21.892±4.502</td>
<td>45.278±3.589</td>
</tr>
<tr>
<td>6</td>
<td>6.483±0.682</td>
<td>64.893±3.494</td>
<td>21.323±8.244</td>
<td>45.397±4.048</td>
<td>66.075±2.962</td>
</tr>
<tr>
<td>8</td>
<td>7.047±0.679</td>
<td>54.228±2.007</td>
<td>13.843±9.231</td>
<td>20.465±4.413</td>
<td>22.175±4.588</td>
</tr>
<tr>
<td>10</td>
<td>8.177±0.491</td>
<td>38.15±1.082</td>
<td>10.474±7.736</td>
<td>16.87±1.571</td>
<td>8.180±4.361</td>
</tr>
<tr>
<td>24</td>
<td>1.890±0.96</td>
<td>8.541±3.68</td>
<td>6.516±4.56</td>
<td>2.122±1.46</td>
<td>1.046±0.89</td>
</tr>
</tbody>
</table>

Values of Plasma glucose are expressed Mean±S.E.M, N=6.

\(^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001\) when compared to control group.

N.S. not significant when compared to control group.

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Table 2. Percentage blood glucose reduction in diabetic rats after treatment with Benincasa hispida

<table>
<thead>
<tr>
<th>Hours</th>
<th>Control</th>
<th>Standard (50mg/kg)</th>
<th>B. hispida (100mg/kg)</th>
<th>B. hispida (200mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>1</td>
<td>1.115±0.644</td>
<td>6.488±3.333 N.S</td>
<td>5.640±3.588 N.S</td>
<td>3.767±1.489 N.S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.450±9.812 N.S</td>
</tr>
<tr>
<td>2</td>
<td>2.815±0.731</td>
<td>16.910±4.838b</td>
<td>17.192±6.491c</td>
<td>48.675±1.785b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>39.6±14.264b</td>
</tr>
<tr>
<td>3</td>
<td>3.945±0.303</td>
<td>28.250±7.498b</td>
<td>41.75±5.915c</td>
<td>51.825±3.261c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>49.625±7.370c</td>
</tr>
<tr>
<td>4</td>
<td>5.932±0.854</td>
<td>44.213±5.971a</td>
<td>48.450±4.197c</td>
<td>53.775±2.664c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>56.050±5.313c</td>
</tr>
<tr>
<td>5</td>
<td>6.483±0.683</td>
<td>64.893±3.494c</td>
<td>52.3±3.141c</td>
<td>54.525±2.8c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61.9±1.807c</td>
</tr>
<tr>
<td>6</td>
<td>7.047±0.679</td>
<td>54.225±7.498b</td>
<td>49.650±2.641c</td>
<td>39.6±1.668c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30.125±1.059c</td>
</tr>
<tr>
<td>7</td>
<td>8.177±0.491</td>
<td>28.153±1.082c</td>
<td>26.425±2.102c</td>
<td>21.425±2.616c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.35±2.908c</td>
</tr>
<tr>
<td>8</td>
<td>8.440±0.652</td>
<td>20.323±2.598b</td>
<td>18.801±1.815c</td>
<td>14.45±4.464 N.S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.25±2.689 N.S</td>
</tr>
<tr>
<td>9</td>
<td>2.142±0.591</td>
<td>2.244±3.653 N.S</td>
<td>8.470±1.890c</td>
<td>4.26±2.581 N.S</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>3.6±1.24 N.S</td>
</tr>
</tbody>
</table>

Values of Plasma glucose are expressed mean±S.E.M, N=6.

*p<0.05,  b*p<0.01,  c*p<0.001 when compared to control group.

N.S. not significant when compared to control group.

Further work is in progress to identify the possible mechanisms of action and to identify the lead molecules responsible for hypoglycemic and anti hyperglycemic activities.

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Antinociceptive and Anti-inflammatory Activities of

*Alstonia scholaris* Linn. R.br.,

Arulmozi.S¹, Papiya Mitra Mazumder², Purnima Ashok¹, Basavaraj Hulkoti¹,

L.Sathiya Narayanan³

¹Department of Pharmacology, K.L.E.S’s College of Pharmacy, II Block, Rajaji Nagar, Bangalore-560 010. Karnataka, India.

²Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi, Jharkhand – 835 215, India.

³Department of Pharmaceutical Chemistry, The Oxford College of Pharmacy, Bangalore – 560 078.

* Author for correspondence E-mail: pharmarul@gmail.com Mobile: 91-9341052889

ABSTRACT - *Alstonia scholaris* linn. (apocynaceae) is traditionally used as an antinociceptive and anti-inflammatory agent. The objective of this study was to investigate experimentally the possible antinociceptive and anti-inflammatory properties of *alstonia scholaris*. The effect of ethanolic extract of leaves of *alstonia scholaris* (EEAS) was evaluated in experimental models of pain and inflammation. The leaf extract at 200 and 400 mg/kg showed significant decrease in acetic acid induced writhings in mice with a maximum of 65.76 % at 400 mg/kg. in hot plate method, the percentage of pain inhibition was found to be 73.90 % and 79.56 % with 200, 400 mg/kg of EEAS respectively. There was a significant (p<0.001) inhibition in carrageenan induced paw edema with EEAS 200 and 400 mg/kg. The anti-inflammatory effects observed with the extract were comparable to that of standard. There was a significant antiulcerogenic property. The present study indicates that the ethanolic extract of *alstonia scholaris* exhibit significant antinociceptive, anti-inflammatory and antiulcerogenic activities.

KEY WORDS: Antinociceptive, Anti-Inflammatory, Alstonia Scholaris, Antiulcerogenic

INTRODUCTION

*Alstonia scholaris* is an antimalarial (1) drug used in the marketed Ayurveda preparation Ayush-64, NRDC, India. The plant *Alstonia scholaris* Linn. R.Br., belongs to the family Apocynaceae and is native of India. It grows throughout India, in deciduous and evergreen forests, also in plains (2).

The bark is bitter, astringent, acrid, thermogenic, digestive, laxative, anthelmintic, febrifuge, antipyretic, depurative, galactogogue, stomachic, cardiotonic and tonic (2). It is useful in fevers, malarial fevers, abdominal disorders, dyspepsia, leprosy, skin diseases, pruritus, tumours, chronic and foul ulcers, asthma, bronchitis, cardiopathy, helminthiasis, agalactia and debility (2, 3). Juice of the leaves and tincture of the bark acts in certain cases as a powerful galactogogue. The drug is also used in cases of snake-bite (2).

The methanolic extract of this plant was found to exhibit pronounced antiplasmodial activity (4). The plant is reported to have anti-mutagenic effect (5). The bark extract of *Alstonia scholaris* has immunostimulating effect. The aqueous extract at low dose induced the cellular immune response while at high dose inhibited the delayed type of hypersensitivity reaction (6). Echitamine chloride, an indole alkaloid, extracted from the bark of *Alstonia scholaris* has got highly promising anticancer (7,8) effect against sarcoma - 180. The plant has hepatoprotective activity on liver injury induced by CCl₄, β-D-galactosamine, acetaminophen and ethanol (9).

In folkllore medicine, milky juice of the plant is applied on wounds, ulcers and rheumatic pains; mixed with oil and dropped into ear, it relieves ear ache (2). Since, the plant is reported to relieve rheumatic pains in folklore medicine (2), it was decided to study the antinociceptive, anti-inflammatory activities of leaf extract of *Alstonia scholaris*. Most of the anti-inflammatory drugs are ulcerogenic. From this viewpoint, in the present study, antiulcerogenic property of *Alstonia scholaris* is also recorded.

MATERIALS AND METHODS

Plant Material and Extraction

The leaves of *Alstonia scholaris* (Family: Apocynaceae) were collected in the month of April May 2006 from hills of Savanthwadi, Maharashtra, India. The plant
material was taxonomically identified by the Botanical Survey of India (BSI), Pune and the voucher specimen AS-1 was retained in herbarium of BSI, Pune for future reference. The dried powdered leaves (500 g) were defatted using petroleum ether and subjected to subsequent extraction in a Soxhlet apparatus by using chloroform and ethanol. The solvents were removed from the respective extracts under reduced pressure to obtain a semisolid mass and vacuum dried to yield solid residues (5.24 % w/w chloroform extract and 6.22 % w/w ethanolic extract). This ethanolic extract of *Alstonia scholaris* is named as EEAS. On preliminary phytochemical screening, EEAS showed positive for the presence of alkaloids, tannins, saponins, glycosides, triterpenoids and flavonoids.

**Animals**

Healthy male Albino Wistar rats weighing between 150-200 g, Albino mice (20 - 25 g) of either sex were maintained in our animal house facility under standard animal house conditions and used for anti-inflammatory and anti-nociceptive activities respectively. CPCSEA guidelines were adhered to during the maintenance and experiment. Experimental protocol was submitted to Institutional Animal Ethics Committee and approval was taken

**Acute Toxicity studies**

Acute toxicity study was carried out for EEAS following OECD guidelines (10). Overnight fasted, healthy Wistar Albino rats (n=3) were administered orally the EEAS in the dose of 2000 mg/kg body weight and observed continuously for 4 h. No visible change was observed in any test animal and all animals survived beyond 24.

**Anti-Nociceptive Activity**

**Hot Plate Method** (11)

The prescreened Swiss Albino mice (reaction time: 2-4 sec) were divided into four groups of six animals each as following: control, standard (Tramadol 5 mg/kg), EEAS 200 mg/kg and EEAS 400 mg/kg. The delay in reaction time (hind paw licking/jumping response) of animals when placed on hot plate maintained at 55 ± 0.1°C (Eddy’s analgesiometer, INCO) was recorded at 0, 30 min, 1, 2, 3 h and tabulated. A cut-off reaction time was fixed at 15 sec to avoid damage to the paws.

**Acetic acid induced writhing** (12)

Swiss Albino mice were assigned into four groups - control, standard (Aspirin 100 mg/kg), EEAS 200 mg/kg and EEAS 400 mg/kg. Writhing was induced after 30 min by intraperitoneal injection of 0.1 ml of 0.6 % acetic acid. The number of wrinkles was counted for 30 min immediately after acetic acid injection in all animals. Percentage protection was calculated for all groups.

**Anti-inflammatory activity**

EEAS was evaluated for anti-inflammatory activity by carrageenan induced rat paw oedema method (13, 14). Male albino Wistar rats (150-200 g) were randomly distributed into 4 groups of 6 animals each. First group served as a control, second group served as the standard (received diclofenac sodium 10 mg/kg, po), while the third and fourth groups received 200 mg/kg, 400 mg/kg body weight of EEAS respectively. After 1 h, 0.1 ml of 1 % w/v suspension of carrageenan was injected into the sub plantar region of left hind paw to all the four groups. The paw volumes were measured using plethysmometer every hour till 6 h after carrageenan injection, and mean increase in paw volumes were noted.

**Anti-Ulcerogenic activity** (15)

Animals of four groups of six rats in each were fasted for 16 h. Control, diclofenac sodium (10 mg/kg), EEAS 200 mg/kg, 400 mg/kg were orally administered. Animals were sacrificed 4 h after the administration of the drugs, the stomachs were removed and cut along the lesser curvature, and the gastric mucosa were washed with normal saline and scored according to the scale. The following scale was used: 0 = no lesion, 0.5 = hyperaemia, 1 = one or two lesions, 2 = severe lesions, 3 = very severe lesions, 4 = mucosa full of lesions. In the second model (16), the above said procedure was followed after administering the respective drugs orally for 7 days.

**Statistical analysis**

The difference in the paw volume at different time intervals and ulcer scores were analysed for statistical significance by performing one-way ANOVA followed by Newmans-Keul multiple comparison test. *p* < 0.05 implies significance.

**RESULTS**

**Hot plate Method**

There was a significant (*p* < 0.01) increase in the basal reaction time on treatment with EEAS throughout the study (Fig 1). EEAS was found to increase the basal reaction time in a dose-dependent manner. The highest nociception inhibition of thermal stimulus was exhibited at 3 h (79.56 %) with 400 mg/kg, which was comparable to the standard (80.02 %).

**Acetic acid induced Writhing**

Dose dependent antinociceptive effect was noted with EEAS at the tested dose levels (Fig 2). Maximum percentage inhibition of writhing response exhibited by the EEAS was at 400 mg/kg (65.76 %) while 200 mg/kg showed 44.30 % reduction in acetic acid induced
**Table 1: Anti-inflammatory effect of EEAS on carrageenan induced paw edema**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
<th>6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.33 ± 0.2108</td>
<td>7.66 ± 0.2108</td>
<td>7.66 ± 0.2108</td>
<td>7.83 ± 0.166</td>
<td>7.66 ± 0.2108</td>
<td>7.33 ± 0.2108</td>
</tr>
<tr>
<td>Standard</td>
<td>4.16 ± 0.16***</td>
<td>4.16 ± 0.16***</td>
<td>4.16 ± 0.16***</td>
<td>4.5 ± 0.2236***</td>
<td>6.33 ± 0.2236***</td>
<td>6.50 ± 0.2236***</td>
</tr>
<tr>
<td>EEAS 200 mg/kg</td>
<td>7.33 ± 0.2108</td>
<td>7.50 ± 0.2236</td>
<td>6.84 ± 0.166</td>
<td>5.66 ± 0.2236***</td>
<td>5.16 ± 0.2108***</td>
<td>6.0 ± 0.2108***</td>
</tr>
<tr>
<td>EEAS 400 mg/kg</td>
<td>7.50 ± 0.2236</td>
<td>6.5 ± 0.166**</td>
<td>4.5 ± 0.166***</td>
<td>4.16 ± 0.16***</td>
<td>5.66 ± 0.2108***</td>
<td>6.0 ± 0.36**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, (n=6). * *p < 0.01, ***p< 0.001 compared to control group (One-way ANOVA followed by Dunnet’s Multiple Comparison test).

**Table 2: Antiulcerogenic activity of EEAS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diclofenac sodium (10 mg/kg)</th>
<th>EEAS 200 mg/kg</th>
<th>EEAS 400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcer Score (4 hrs)</td>
<td>0.26 ± 0.10</td>
<td>3.33 ± 0.34***</td>
<td>0.26 ± 0.12</td>
<td>0.38 ± 0.82</td>
</tr>
<tr>
<td>Ulcer score (7 days)</td>
<td>0.25 ± 0.12</td>
<td>3.84 ± 0.16**</td>
<td>0.54 ± 0.14</td>
<td>0.66 ± 0.22</td>
</tr>
</tbody>
</table>

Ulcer scores: 0 = no lesion, 0.5 = hyperaemia, 1 = one or two lesions, 2 = severe lesions, 3 = very severe lesions, 4 = mucosa full of lesions.

Values are mean ± SEM, (n=6). * *p < 0.01, ***p< 0.001 compared to control group (One-way ANOVA followed by Dunnet’s Multiple Comparison test).

**Figure 1: Antinociceptive effect of EEAS on thermal stimulation**

*p< 0.05, * *p< 0.01, compared to control group (One-way ANOVA followed by Dunnet’s Multiple Comparison test).
Figure 2: Antinociceptive effect of EEAS on acetic acid induced writhing

* *p< 0.01 compared to control group (One-way ANOVA followed by Dunnet’s Multiple Comparison test).

writhe response, which was comparable to that of standard that caused 68.78 % inhibition.

**Anti-inflammatory activity**

EEAS showed significant (p< 0.01) decrease in paw oedema after 3 h of injection of carrageenan (Table 1). However the effect was more prominent (p< 0.01) at 4, 5 and 6 h. The percentage inhibition of paw volume of EEAS 400 mg/kg was comparable to that of standard at 4 h and more prominent at 5 h and 6 h.

**Antiulcerogenic activity**

The groups of animals treated with EEAS did not show ulceration in the stomach after 16 h of fasting, whereas the ulcer score was found to be significantly high (p<0.01) in rats administered dicyclofenac sodium (Table 2). Treatment of the extracts for seven days did not show any ulceration whereas the ulcer score was significantly (p<0.01) high with diclofenac sodium treated rats (Table 2).

**DISCUSSION**

The thermal stimuli in hotplate test and the writhing response of the animals to an intra-peritoneal injection of noxious chemical are used to screen both peripherally and centrally acting analgesic activity. Acetic acid causes algesia by liberating endogenous substances that excite the pain nerve endings (17). From the results it is apparent that the EEAS showed a significant antinociceptive effect in hot plate test and writhing response, which are comparable to that of the standard. Studies demonstrate that various flavonoids such as rutin, quercetin, luteolin, hesperidin and biflavonoids produced significant antinociceptive and anti-inflammatory activities (18, 19). There are also few reports on the role of tannins in antinociceptive and anti-inflammatory activities (20). NSAIDs can inhibit cyclo-oxygenase in peripheral tissues, thus interfering with the mechanism of transduction in primary afferent nociceptors (21). The mechanisms of antinociceptive action of EEAS could be due to the presence of flavonoids and mediated through central and peripheral mechanisms. Carrageenan induced paw oedema was taken as a prototype of exudative phase of acute inflammation. Inflammatory stimuli microbes, chemicals and necrosed cells activate the different mediator systems through a common trigger mechanism. The development of carrageenan-induced oedema is believed to be biphasic. The early phase is attributed to the release of histamine and serotonin (22, 23) and the delayed phase is sustained by the leucotrienes and prostaglandins (24). Flavonoids and tannins are reported to inhibit PG synthesis (25). As phytochemical tests showed presence of triterpenoids, tannins and flavonoids in EEAS, it might suppress the formation of PG or antagonize their action and exert the activity. A
strong correlation between the potency of NSAIDs as an inhibitor of prostaglandin (PG) synthesis and ulcerogenic activity has been suggested (26). Most of the NSAIDs have well-balanced anti-inflammatory and ulcerogenic activities, which are considered to be due to PG synthetase inhibitor activity. The ethanolic extract of *Alstonia scholaris* possesses a marked anti-inflammatory activity and its lack of ulcerogenic activity is suggestive that it does not act mainly by PG synthetase inhibition, (but through some selective mechanism viz. Cox-2). Further, chronic administration of the extracts did not produce ulcer which proves the safety of the extracts. This is a point of distinct advantage when considering the chronic administration.

**CONCLUSION**
The ethanolic extract of *Alstonia scholaris* Linn. comprises antinociceptive and anti-inflammatory activities. EAS, whose spectrum of anti-inflammatory activity appears to be different from classical NSAIDs, with the distinct advantage of its freedom from gastric ulcerogenic effects, is likely to have therapeutic potential. It is worthwhile to isolate the bioactive principles, which are responsible for these activities, which is in process. Studies are underway to evaluate the anti-arthritic property of the extract. However, further studies are essential to elucidate the detailed mechanisms of action for antinociceptive and anti-inflammatory activities.

**ACKNOWLEDGEMENTS**
The authors wish to thank Prof. B.G.Desai, Director, K.L.E. Society’s college of Pharmacy, Bangalore and Department of Pharmaceutical Sciences, Birla Institute of Technology, Ranchi for the facilities provided for the study.

**REFERENCES**


PHCOG MAG.: Research Article

Lipid lowering effect of aqueous leaves extract of *Murraya koenigii* (curry leaf) on alloxan-induced male diabetic rats

M. K. Vinuthan\textsuperscript{a}, V. Girish Kumar\textsuperscript{a}, M. Narayanaswamy\textsuperscript{a} and T. Veena\textsuperscript{b}

\textsuperscript{a}Department of Physiology, \textsuperscript{b}Department of Biochemistry, Veterinary college, KVAFSU, Bangalore-560024, Karnataka, India.

Correspondence: drvinuthanmk@rediffmail.com

ABSTRACT - *Murraya koenigii* leaves is considered extensively in the indigenous system of medicine as an antidiabetic agent in many Asian countries. The current investigation focuses attention on the lipid lowering property of the aqueous extract of *Murraya koenigii* leaves on experimentally induced diabetes in rats. The lipid parameters studied are plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), very low density cholesterol (VLDL-C), triglyceride (TG) and phospholipids (PL). Extracts were orally administered daily for 8 weeks at dose of 600 mg/kg in alloxan diabetic rats. The levels of TC, LDL-C, HDL-C, VLDL-C, triglyceride and phospholipids were reduced significantly (P<0.05), while HDL-C levels did not alter significantly in rats given, aqueous extracts of *Murraya koenigii* when compared to diabetic control rats. In conclusion, these results showed that *Murraya koenigii* leaves extract when administered orally, can reduce plasma lipids level. These results further suggest that *Murraya koenigii* may be useful in the therapy and management of diabetic hyperlipidemia through reducing lipid levels.

KEY WORDS: alloxan, diabetes mellitus, hyperlipidemia, *Murraya koenigii*.

INTRODUCTION

Diabetes mellitus is a syndrome resulting from a variable interaction of hereditary and environmental factors and characterized by depleted insulin secretion, hyperglycaemia and altered metabolism of lipids, carbohydrates and proteins, in addition to damaged β-cells of pancreas and an increased risk of complications of vascular diseases (1). A number of pharmacological and chemical agents act as diabetogenic and produce variety of diabetic complications. Alloxan induction of diabetes is an experimental model widely used to study glycemic and lipidemic changes in plasma. Many species of plants and herbs are known to act as anti-diabetic agents, but only a few of them have been investigated (2).

*Murraya koenigii* (family, Rutaceae), popularly known as curry leaf is a medicinal plant that grows throughout the greater parts of India and South East Asia (3). This species is known to possess anti-inflammatory, antidyserteric, antioxidant, antidiabetic and diverse pharmacological properties (4-6). Several studies also shown that curry leaf decreased blood glucose significantly in different animal models (7-9). *Murraya koenigii* leaves have been shown to prevent hyperglycaemia and pancreatic damage induced by alloxan in rats (10). Dietary supplement with curry leaves has been shown to reduced total serum cholesterol, LDL + VLDL, increased HDL, decreased the release of lipoproteins into circulation and increased catalase activity in rats (11). The present study was carried out in Sprague-Dawley rats to explore the effects of *Murraya koenigii* aqueous leaf extract on plasma lipid profile changes associated with diabetes.

MATERIALS AND METHODS

Plant material: Leaves of *Murraya koenigii* were collected fresh from plants grown in University of Agricultural Sciences campus, Bangalore, Karnataka, India. Taxonomic identification was authenticated by the Department of Botany, University of Agricultural Science, Bangalore, Karnataka, India. The leaves were air dried, reduced to powder and were kept separately in airtight containers until the time of use.

Preparation of aqueous extract

Powder of *Murraya koenigii* leaves (100 g) was taken and 200 ml of distilled water was added and boiled and later it was filtered off by using filter. The final concentration of the extract was 150 mg/ml. The filtrate obtained served as crude extract and administered orally into the experimental animals at the concentration of 600 mg/kg body weight per day for 8 weeks.

Animals

Experimental animals and induction of diabetes

Adult male Sprague-Dawley rats (200-300 g) bred in the Central Animal House, University of Agricultural Sciences campus, Bangalore, were used in this study. The animals were housed in polypropylene cages under
controlled conditions of 12-h light/dark cycle, and at
24 ± 2°C. They were maintained on standard pellet
diet containing 22% protein, 4.28% oil, 3.02% fiber,
7.8% ash and 1.3% silica (Amrut Laboratory Animal
Feed, Nav Maharashtra Chakan Oil Mill Ltd, Pune,
India) and water ad libitum.
Diabetes was induced in rats by intraperitoneal
injection of 100 mg/kg body weight of alloxan
monohydrate (5% w/v), freshly dissolved in
physiological saline immediately before use (12-14).
The diabetic state was confirmed 48 h after alloxan
injection by weight loss, glucosuria (15) and
hyperglycemia (16) and the animals, which presented
blood glucose level above 200 mg/dl, as well as with
the clinical signs of polydipsia, polyuria and
polyphagia were selected for the experiment.

Treatment
Animals were divided into following 3 groups of 6
each. Group I: Normal rats received only physiological
saline, Group II: Control diabetic rats received only
physiological saline, Group III: Diabetic rats received
aqueous extract of *Murraya koenigii* leaves (600 mg/kg
body weight) per orally daily. This study was carried
out for 8 weeks according to the guidelines of the
Institutional Animal Ethical Committee.

Animals were anaesthetized with ether after which
blood from retro orbital venous plexus was collected
for estimation of plasma lipid profile.

**Biochemical analysis**
Plasma TC (17), HDL-C (17) and TG (18) estimation
were carried out using respective diagnostic
commercial kits from Accurex Biomedical Pvt. Ltd.,
Bombay, India. Phospholipids (19) level was estimated
in plasma. VLDL-C and LDL-C in plasma were also
calculated as per Friedewald's equation (20).

**Statistical analysis**
The results were expressed as mean ± SEM.
Statistically analysis was carried out using one-way
ANOVA followed by Bonferroni's test. Differences
below P<0.05 implied significance (21).

**RESULTS AND DISCUSSION**
Table 1a & 1b. Shows the effect of oral administration
of aqueous extract of *Murraya koenigii* on plasma
lipids. The rats of Group II, diabetic control showed a
marked increase in plasma TC, LDL-C, VLDL-C, TG and
PL and a fall in HDL-C levels when compared to normal
control group. However, following treatment with
aqueous extract of *Murraya koenigii* (600 mg/kg) for 8
weeks, the plasma TC, LDL-C, VLDL-C, TG and PL were
reduced significantly (P<0.05), while HDL-C remains
unchanged in extract treated group when compared to
diabetic control group.

**Table 1a. Effect of aqueous extract of *Murraya koenigii* on the lipid profile of alloxan diabetic male rats.**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>TC</th>
<th>TG</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>71.48 ± 0.82</td>
<td>78.43 ± 1.35</td>
<td>75.95 ± 1.07</td>
</tr>
<tr>
<td>Group II</td>
<td>94.69 ± 2.23*&lt;sup&gt;a&lt;/sup&gt;</td>
<td>155.10 ± 2.17*&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126.00 ± 2.15*&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>80.18 ± 3.01*&lt;sup&gt;b&lt;/sup&gt;</td>
<td>125.10 ± 6.33*&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.80 ± 3.59*&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 1b. Effect of aqueous extract of *Murraya koenigii* on the lipid profile of alloxan diabetic male rats.**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>LDL-C</th>
<th>HDL-C</th>
<th>VLDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>25.13 ± 1.30</td>
<td>43.36 ± 0.73</td>
<td>19.73 ± 0.63</td>
</tr>
<tr>
<td>Group II</td>
<td>28.51 ± 1.34*&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.62 ± 0.46*&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.04 ± 0.43*&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
<td>25.38 ± 1.40*&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.83 ± 0.41</td>
<td>25.02 ± 1.27*&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are expressed in mg/dl. Values are expressed as mean ± SEM for six animals in each group.
*<sup>a</sup> values are significantly different from normal control (Group I) rats.
*<sup>b</sup> values are significantly different from diabetic control (Group II) rats.
Bonferroni’s test (<0.05) was used ; Group I (normal control rats), Group II (diabetic control rats), Group
III (aqueous extract treated diabetic rats), TC - total cholesterol, TG - triglycerides, PL - phospholipids,
LDL-C - LDL-cholesterol, HDL-C - HDL-cholesterol, VLDL-C - VLDL-cholesterol
Studies in human and animals demonstrated that alteration of blood lipid profiles in condition of diabetes represents a risk factor for cardiovascular diseases (22). A number of pharmacological and chemical agents act as diabetogenic and produce variety of diabetic complications. Alloxan induction of diabetes is an experimental model widely used to study glycemic and lipidemic changes in plasma. Previous study demonstrated that aqueous extract of *Murraya koenigii* had a hypoglycemic effect in diabetic rats (10). The present study evaluated the effect of *Murraya koenigii* aqueous leaf extract on lipid parameters such as plasma TC, LDL-C, VLDL-C, HDL-C, TG and PL in alloxan-induced experimental diabetic rats.

Following the treatment with alloxan to rats a remarkable rise in the levels of plasma TC, LDL-C, were observed. Previous reports suggest that, elevated TC and LDL-C levels in the plasma of diabetic are considered to be a prime cause of coronary heart disease (CHD) (23-25). Many epidemiological studies showed that drug or diet induced reduction of TC and LDL-C could reduce the risk of CHD (26-28). In the present study, its recovery towards normal levels in aqueous extract administered diabetic rats coincides with the above observations, thus unearthing the cardioprotective effect of *Murraya koenigii*. The TG, PL and VLDL-C content in plasma registered a significant hike in diabetic control group, which was retrieved to near normalcy in aqueous extract treated diabetic rats. This observation also indicates the lipid lowering potential of *Murraya koenigii*.

Phytochemical analysis of *Murraya koenigii* leaf shows the presence of alkaloids, flavonoids, glycosides, minerals, vitamins and many other compounds (3, 29, 30). It has been reported the effect of tertiary and quaternary alkaloids, flavonoids and glycoside components reduces lipid levels in animals (31, 32). The varied chemical composition found in this leaf extract assigns to its lipid lowering property. This property of *Murraya koenigii* leaf extract may also be because of its other properties like antiinflammatory property which may prevent inflammatory pancreatic damage, immunomodulating property and antioxidant property (33) thereby reducing the oxidative stress imposed by the chemicals (alloxan); this antioxidant mechanism seems to be important as *Murraya koenigii* leaves has been shown to reduce oxidative stress (6) and oxidative stress has been found to be the most important mechanism in diabetic condition.

In conclusion, this study has shown that, oral administration of the aqueous extract of *Murraya koenigii* leaves have significantly reduced plasma lipid levels associated with diabetes mellitus. Thus it can be concluded that extract of *Murraya koenigii* leaves prevents as well as reverse the plasma lipid profile, thus emphasizing the protective role against diabetes induced hyperlipidemia. Further studies on the active components of *Murraya koenigii* and mechanism(s) of its protective effect against diabetic hyperlipidemia are needed.

REFERENCES


PHCOG MAG.: Research Article

Study of antacid and diuretic activity of ash and extracts of Musa sapientum L. fruit peel.

D. L. Jain a*, A. M. Baheti b, S. R. Parakh b, S. P. Ingale b, P.L. Ingale b

a Technical University Munich, Germany.
b MAEER’s Maharashtra Institute of Pharmacy, MIT campus, Paud Road, Pune- 411038, India.
E-mail address for correspondence: suvarnaingale@gmail.com

ABSTRACT
The antacid and diuretic activity of different extracts and ash of peels of Musa sapientum L. (Musaceae) in rat was studied. The study suggested that the ash has good antacid activity. Diuretic study was carried out as per Lipschitz et al., (1943), where successive aqueous, Ethanolic and Petroleum ether extracts and ash of peel of Musa sapientum L. were studied for diuretic activity. The 6 hrs acute study of successive aqueous, Ethanolic extracts and ash of the peel showed increase in urine volume and K+ ion excretion as compared to normal saline. Urinary levels of sodium, potassium (by flame photometry) and chloride (by titrimetry) were estimated. On the basis of observed results it is concluded that ash might have good antacid activity while successive ethanolic extract and ash exhibited evidence of diuretic potentials in normal rat in our experimental model. The histopathological examination and toxic effects on vital organs remained to be studied before their recommendation for use as diuretic agents.

KEY WORDS - Antacid; Diuretic; Flame photometry; Musa sapientum; Musaceae.

INTRODUCTION
Musa sapientum Linn (Musaceae) commonly called ‘Kela’ in Hindi (English: Banana) is extensively cultivated in northern part of Maharashtra. It is one of the most popular fruit crop in India and possesses many curative properties and prevent many kinds of illnesses and conditions. Different parts of plant are used very frequently in different worship ceremonies by the Indians every part of the tree is being used for some purpose like food, fuel or timber (1). It is perennial herbaceous plant, grows to 5-9 m in height. It has tuberous subterranean rhizome, from which the leaves are folded within each other producing false stem, from which the long, narrow blades protrude and spread out. In the center of the folded leaf sheaths, a growing point forms the top of rhizomes, grows up and emerges as an overhanging inflorescence with a succession of reddish brown bracts. The bracts unfold from the base to the tip and fall off. Within the lower 1-12 bracts arise 14-18 female flowers in double rows these develop into fruits (2). The roots are adventitious. The leaves are large from 1.5-3.5 meters and 0.6 meter wide (3).

In India hot water extract of dried fruits, flowers and roots is used orally for diabetes, the dried flower along with dried fruits of Coccinia indica is used to prevent conception orally. The roots are used as anthelmintic, aphrodisiac, laxative and tonic. The fresh fruit is used for peptic and duodenal ulcers. The leaf ash is mixed with honey and taken orally for cough. Ripe plantain is emollient, demulcent and nutrient. Unripe plantain is cooling and astringent. Fully ripe fruit have laxative effect while flowers are used as astringent. Root is anthelmintic and a valuable alternative (4, 2). The peels of Musa sapientum plant are used in Jalgaon and its environment as antacid and diuretic agent. But adequate characterization of its antacid and diuretic activity has not been yet performed. Also such reports are not available in the literature though the activity is reported. The present study was undertaken for scientific evaluation of antacid (in vitro) activity using Acid-neutralizing capacity test USP29 and diuretic (in vivo) activity in normal healthy rats.

MATERIALS AND METHODS

Plant material
The fresh banana fruits were procured from local farmers in the Pune region and identified correctly by Pharmacognosy Department of MAEER’S Maharashtra Institute of Pharmacy, Paud Road, Pune-411038, India. The peels of the fruits were dried and powdered using laboratory grinder.

Drugs
Furosemide and urea were procured from Himedia Laboratories, Mumbai, while, Gokharu khada (Diuretic preparation, as described in Ayurveda) was purchased.
from market (manufacturer: Ayurved Rasashala, Pune, India). All other reagents were analytical grade.

**Preparation of the extracts**

Fresh banana peels were extracted with decreasing polarity of solvents to obtain successive extracts using pet ether, benzene, chloroform, ethanol and water. The extracts were prepared using soxhlet apparatus as one part of the material with three parts of solvent for 3 hrs. This was repeated twice with fresh solvent. The extracts from both washes were pooled and concentrated under vacuum at 60\(^\circ\) C to obtain a dry extract. The marc obtained following the pet ether extraction was later extracted with benzene and dried in same manner as above. The same procedures were followed using chloroform, ethanol (95%) and water to obtain their successive extracts. All the extracts were stored at room temperature in tightly closed containers.

**Preparation of ash**

The ash was prepared by incineration of the peels into the furnace at 800\(^\circ\) C for 1.5 hrs and cooling at room temperature. Fine powder was prepared using laboratory grinder and passed through sieve no. 120.

**Animals**

The male Wistar rats weighing 160-200 gm were used to study the diuretic activity. The animals were housed under standard environmental conditions (22±3\(^\circ\) C, 55±5 % humidity and a 12 h light/ dark cycle) and fed with standard rodent diet and water *ad libitum*. The institutional Animal Ethical Committee approved all the experimental protocols.

**Toxicological study**

The male albino rats of Wistar strain weighing 160-200 gm were divided into different groups comprising of six animals each. The control group received normal saline 25ml/kg i.p. The other groups received 100, 200, 400, 600, 800, 1000, 2000, 3000 and 4000 mg/kg of test extracts. The animals were observed continuously for the behavioural changes for the first 4 hours and then observed for mortality if any for 24 hours. (5)

**Acid-Neutralizing Capacity USP**

The Acid-Neutralizing capacity was carried out as per USP29. In short, all tests were conducted at temperature 37± 3\(^\circ\) C. A pH meter was standardized using the 0.05M potassium biphthalate and 0.05M potassium tetraoxalate standardized buffers. Magnetic stirrer was used to produce stirring rate 300s: 30rpm. 0.5 gm of each ash, pet ether, successive ethanolic and successive water extracts were transferred to 250 ml beaker and 70 ml distilled water was added. It was mixed with magnetic stirrer for 1 min. Then 30 ml 1.0N HCl was added to the test solutions with continuous stirring for 15 min. Excess HCl was titrated with 0.5 N NaOH to attain a stable pH of 3.5. The number of mEq of acid consumed was calculated by formula:

\[
\text{Total mEq} = (30 \times N_{\text{HCl}}) \times (V_{\text{NaOH}} \times N_{\text{NaOH}})
\]

Where \(N_{\text{HCl}}\) and \(N_{\text{NaOH}}\) are normalities of hydrochloric acid and sodium hydroxide respectively and \(V_{\text{NaOH}}\) is volume of sodium hydroxide and the result were expressed as total mEq per gm of substance(6).

**Diuretic study (Lipschitz method)**

Diuretic activity was carried out as per the method of Lipschitz et.al. (1943)7, 8). In brief, ash, pet ether, successive ethanol (95%) and successive aqueous extracts were subjected to diuretic study. The screening was performed on healthy rats (160-200 gm). Frusemide (20 mg/kg), urea (500 mg/kg) and Gokharu khada (500 mg/kg) were used as reference standards and were dissolved in saline solution for administration while normal saline (25 ml/kg) was used as vehicle. The rats were divided in 12 groups each containing 6 rats (n = 6). Rats were kept for fasting for 18 hrs before the study. The control group received normal saline and test groups received different extracts (500 and 1000 mg/kg) and ash (500 and 1000 mg/kg) dissolved in normal saline. The doses of extracts were decided on the basis of acute toxicity study. The doses were given by oral route and rats were kept in specially designed metabolic cages for the collection of urine for 6 hrs. The urine volume during 6 hrs is measured and urine electrolyte estimation was carried out for Na\(^+\), K\(^+\) using flame photometer (9) and Cl\(^-\) was estimated by titration (8,10,11).

**Statistical analysis**

All results are expressed as mean ± standard error. The data was analyzed statistically using ANOVA followed by Dunnett’s Multiple Comparison Test.

**RESULTS**

**Extraction**

The florescence study showed characteristic color changes with solvents like pet ether, benzene and ethanol (95%). The obtained yields are given in Table 1. Since the yields of successive extract of benzene and chloroform were insignificant so further studies for these extracts were discontinued and only pet ether, successive ethanol (95%) and successive aqueous extracts were subjected to further studies.

**Acid-Neutralizing Capacity Test USP29:**

The ash complied with the Acid-Neutralizing Capacity Test and pH after 10 min was 7.8. Ash neutralized 10.6 mEq of acid whereas pet ether, ethanol and aqueous extracts were unable to neutralize acid.
TABLE 1: Estimated yields % using different solvents.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvent</th>
<th>Estimated yields % (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pet ether</td>
<td>2.41</td>
</tr>
<tr>
<td>2</td>
<td>Benzene</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>Chloroform</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>Ethanol (95%)</td>
<td>1.36</td>
</tr>
<tr>
<td>5</td>
<td>Water</td>
<td>0.36</td>
</tr>
</tbody>
</table>

**Toxicological study**

Neither mortality nor any gross behavioural changes were observed during and after the treatment. The ash, ethanolic and aqueous extracts were found to be safe up to 2000 mg/kg.

**Diuretic activity**

Frasemide treated rats showed a significant increase in volume of urine and urinary excretion of sodium, potassium and chloride (p < 0.01) as compared to control while urea treated rats did not show any significant increase in urine volume but has high electrolyte excretion potential (p < 0.01). Higher electrolyte excretion (p < 0.01) was observed in ayurvedic diuretic preparation, Gokharu Kadha but not significant increase in urine volume. The successive aqueous extract was unable to produce significant actions in dose of 500 mg/kg but at high dose of 1000 mg/kg successive aqueous extract showed significant increase in volume of urine and also urinary excretion of sodium, potassium and chloride. The successive ethanolic extracts has shown diuretic activity (p<0.01) wherein significant increase in K⁺ but not in Na⁺ excretion when compared to control was observed. Pet. ether extract did not show remarkable increase in volume of urine, urinary sodium, potassium or chloride. It was observed that ash also increased diuresis and urinary excretion of electrolyte (p < 0.01). The results are summarized in Table 2.

**DISCUSSION**

Diuretics relieve pulmonary congestion and peripheral edema. These agents are useful in reducing the syndrome of volume overload, including orthopnea and paroxysmal nocturnal dyspnoea. They decrease plasma volume and subsequently venous return to the heart (preload). This decreases cardiac workload, oxygen demand and plasma volume, thus decreasing blood pressure (12). Thus, diuretics play an important role in hypertensive patients. In present study, we can demonstrate that ash and successive aqueous and ethanol extract may produce diuretic effect by increasing the excretion of Na⁺, K⁺ and Cl⁻.

The control of plasma sodium is important in the regulation of blood volume and pressure; the control of plasma potassium is required to maintain proper function of cardiac and skeletal muscles (13). The regulation of Na⁺/ K⁺ balance is also intimately related to renal control of acid-base balance. The K⁺ loss that occurs with many diuretics may lead to hypokalemia. For this reason, generally potassium-sparing diuretics are recommended (14). In present study, ash and successive aqueous and ethanol extract showed elevated levels of K⁺ in urine, which may increase risk of hypokalemia, and hence its potassium sparing capacity has to be investigated.

Active phytoprinciples such as flavonoids, saponins and terpenoids are known to be responsible for diuretic activity (15,16,17). These active principles in successive aqueous and ethanolic extract may be responsible for diuretic activity. Isolation of these active principles and study of their exact mechanism of action needs to be investigated.

Results of present investigation showed that ash is most effective in increasing urinary electrolyte concentration of all the ions i.e. Na⁺, K⁺ and Cl⁻ followed by successive aqueous and ethanol extracts while petroleum ether extract did not show significant increase in urinary electrolyte concentration.

In the in vitro studies simulated to compare the antacid effects, ash showed good antacid activity while others did not. Antacids are weak bases that react with gastric acid to form water and salt thereby diminishing gastric acidity. Antacid products vary widely in their chemical composition, acid neutralizing capacity, sodium content and palatability. Acid neutralizing capacity depends upon capacity to neutralize gastric HCl and the extent of food contents in stomach (12). It was observed that 0.5 gm of ash neutralized 10.6 mEq of HCl acid in Acid Neutralizing Capacity Test USP29. These beneficial effects observed under in vitro simulations remains to be investigated in vivo as well as at the biochemical level to understand the mechanisms involved.
Table 2: Diuretic activity of successive aqueous, ethanolic and pet. ether extracts of Musa sapientum.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (n= 6)</th>
<th>Volume of Urine (ml/ 6 hrs)</th>
<th>Sodium (mMol/l)</th>
<th>Potassium (mMol/l)</th>
<th>Chloride (mMol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal saline (25 ml/ kg)</td>
<td>0.74±0.19</td>
<td>98.3±11</td>
<td>52.3±8.9</td>
<td>101.2±8.85</td>
</tr>
<tr>
<td>II</td>
<td>Frusemide (20 mg/ kg)</td>
<td>3±0.34*</td>
<td>137±7.6*</td>
<td>96.64±10.2*</td>
<td>153±13.2*</td>
</tr>
<tr>
<td>III</td>
<td>Urea (500 mg/ kg)</td>
<td>0.85±0.13</td>
<td>112.1±11.9*</td>
<td>78.7±7.9*</td>
<td>121.3±14.1*</td>
</tr>
<tr>
<td>IV</td>
<td>Gokharu Kadha (500 mg/kg)</td>
<td>0.71±0.18</td>
<td>118.3±10.4*</td>
<td>87.3±8.4*</td>
<td>131.2±9.3*</td>
</tr>
<tr>
<td>V</td>
<td>SAE (500 mg/kg)</td>
<td>1±0.17</td>
<td>99.7±8.4</td>
<td>114.7±9.8*</td>
<td>122±14.2*</td>
</tr>
<tr>
<td>VI</td>
<td>SAE (1000 mg/kg)</td>
<td>1.5±0.25*</td>
<td>110.7±2.3**</td>
<td>133±7.3*</td>
<td>157.7±12.6*</td>
</tr>
<tr>
<td>VII</td>
<td>SEE (500 mg/kg)</td>
<td>1.09±0.18*</td>
<td>98.6±5.3</td>
<td>110.7±6.9*</td>
<td>115.2±10.3**</td>
</tr>
<tr>
<td>VIII</td>
<td>SEE (1000 mg/kg)</td>
<td>1.42±0.10*</td>
<td>100.2±1.3</td>
<td>120.8±3.5*</td>
<td>131.1±13.2*</td>
</tr>
<tr>
<td>IX</td>
<td>Ash (500 mg/kg)</td>
<td>1.12±0.20*</td>
<td>100.8±0.3</td>
<td>90.2±6.8*</td>
<td>120.3±7.6*</td>
</tr>
<tr>
<td>X</td>
<td>Ash (1000 mg/kg)</td>
<td>2.44±0.39*</td>
<td>119.1±13.3*</td>
<td>104.4±7.5*</td>
<td>133.4±9.7*</td>
</tr>
<tr>
<td>XI</td>
<td>PEE (500 mg/kg)</td>
<td>0.69±0.12</td>
<td>90.8±9</td>
<td>52.7±8.2</td>
<td>98.7±7.7</td>
</tr>
<tr>
<td>XII</td>
<td>PEE (1000 mg/kg)</td>
<td>0.73±0.17</td>
<td>92.1±8.3</td>
<td>51.9±7.6</td>
<td>97.7±7.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, * p< 0.01, ** p< 0.05 when compared to normal saline (control)

Note: SAE: Successive Aqueous Extract, SEE: Successive Ethanolic Extract, PEE: Pet Ether Extract.

CONCLUSION

On the basis of the results of present investigation, we can conclude that ash and successive ethanolic extracts might be good diuretics. In present study, no lethality was observed at least for the dose and duration used. However, advanced toxicological studies remain to be performed in mice and rats. It remains necessary to study eventual adverse effect(s) of this plant such as alteration of some neural, metabolic and hormonal parameters. Future studies play an important role before its recommendation to clinical use. The precise site(s) and the molecular and cellular mechanism(s) of this ash and successive ethanolic extract action remain to be elucidated. Also, the selective isolation or activity-guided isolation along with deciphering the chemical nature of the compounds will help further to elucidate the exact mechanism(s).

REFERENCES


PHCOG MAG.: Research Article
Hepatoprotective activity of *Cleome viscosa* against Carbon tetrachloride induced hepatotoxicity in rats
Sengottuvelu S.*, Duraisamy R., Nandhakumar J. and Sivakumar T.

*Nandha College of Pharmacy, Koorapalayam Privu, Erode-52, Tamil Nadu, India.*

*Corresponding author. Tel.: +424-2253045 E-mail address: sengt@rediffmail.com*

**ABSTRACT** - The present study was conducted to evaluate the hepatoprotective activity of aqueous seed extract of *Cleome viscosa* (CV) against carbon tetrachloride (CCL₄) induced liver damage in wistar rats. The aqueous seed extract of CV (200 mg/kg) was administered orally to the animals with hepatotoxicity induced by CCL₄. Silymarin (200mg/kg) was given as reference standard. The seed extract was effective in protecting the liver against the injury induced by CCL₄ in animals. This was evident from significant reduction in serum enzyme aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase and lipid peroxidase and increase in reduced glutathione (GSH). Various pathological changes like steatosis, centrilobular necrosis and vacuolization observed in CCL₄ treated rats, which were prevented to a moderate extent in groups, treated with *Cleome viscosa* and silymarin. It was concluded from the study that aqueous seed extract of CV possesses hepatoprotective activity against CCL₄ induced hepatotoxicity in rats.

**KEYWORDS:** Carbon tetrachloride; *Cleome viscosa*; Hepatoprotective activity; Silymarin.

**INTRODUCTION**
Liver disease remains one of the serious health problems. Herbs play a major role in the management of various liver disorders. A number of plants possess hepatoprotective property (1). *Cleome viscosa* (CV) is a common weed used extensively in the Indian traditional system. It is an annual, sticky herb belonging to the family Capparaceae. The plant distributed throughout the plains in India. The aqueous extract of the seeds of this plant has traditionally been used for the treatment of various liver disorders and as analgesic. It potentiates the barbiturate sleeping time in rats and also had a mild laxative effect (2). The leaves of CV were reported to possess hepatoprotective activity in rats (3), based on the above, the present study has been undertaken to investigate the hepatoprotective activity of aqueous extract of the seeds of CV against CCL₄ induced hepatic damage in rats.

**MATERIALS AND METHODS**

**Animals**
AMale Wistar Albino rats weighing between 150 - 250 gm were used. The animals were obtained from animal house, IRT Perundurai medical college, Erode, India. On arrival the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2°C and relative humidity of 30 - 70 %. A 12:12 light:day cycle was followed. All animals were allowed to free access to water and fed with standard commercial rat chaw pallets (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics Committee (Regd no: 688/2/C-PCEA) and were in accordance with the guidelines of the CPCSEA.

**Preparation of seed extract**
The seeds of *Cleome viscosa* were collected from mature plants during the month of October from the outskirts of Erode city. The plant was authenticated by the botanist of Botanical Survey of India, Agricultural University, Coimbatore. The collected seeds were sun dried for 7 days and ground to coarse powder using a blender. The powdered seeds were soaked in sufficient quantity of purified water for maceration, after maceration the meristum was collected, filtered and then evaporated to obtain dry extract and it was used for the study.

**Drugs and chemicals**
CCL₄ was obtained from S.D. Fine-chem. Ltd. Boisar, and Silymarin from Indena Spa. Milan, Italy. All other chemicals were obtained from local sources and were of analytical grade.

**Methodology**
A total of 24 animals were equally divided into 4 groups of six each. Group - I served as normal control received 0.3% carboxy methyl cellulose (1 ml/kg p.o.) once daily for 7 days. Group - II received equal mixture...
of CCl₄ and olive oil (50 % v/v, 0.5 ml/kg i.p.) once daily for 7 days (4). Group - III received equal mixture of CCl₄ and olive oil and CV extract (200 mg/kg p.o.) simultaneously for 7 days. Group - IV received equal mixture of CCl₄ and olive oil and standard drug silymarin (200 mg/kg p.o.) (5), simultaneously for 7 days. On 8th day the blood was collected by direct cardiac puncture under light ether anaesthesia and serum was separated for different biochemical analysis. All animals were sacrificed by cervical decapitation and immediately, the livers were dissected out, washed in the ice cold saline and homogenate was prepared in 0.05 M sodium phosphate buffer (pH 7.0) and centrifuged. The supernatant was used for the estimation of γ-glutaryl transpeptidase, lipid peroxidase and reduced glutathione (GSH).

**Enzyme assays**

The activities of serum hepatic marker enzymes namely aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) (6,7) were assayed in serum using standard kits from Lupin Laboratories and pointe scientifics. The results were expressed as units/litre (U/L). γ - glutamy transpeptidase activity was assayed by the method of Tate and Meister (8). GSH was estimated in the liver homogenate using DTNB by the method of Buetler (9). The absorbance was read at 412 nm and the results were expressed as mg GSH/g of wet tissue. The lipid peroxidation in the liver was determined by the method of Ohkawa (10).

**Histopathological examination**

A portion of liver tissues were collected in 10 % formaldehyde solution for histopathological studies. They were processed in an automatic tissue processor and embedded in paraffin wax. Sections of 5µm were cut on a rotary microtome by serial sectioning until the entire thickness of the liver was sectioned. Staining was done by haematoxylin and eosin and later the microscopic slides of the liver cells were photographed.

**Statistical analysis**

The values were expressed as mean ± SEM. The statistical analysis was carried out by one way analysis of variance (ANOVA). P values <0.05 were considered significant.

**RESULTS**

The results of transaminase are in table 1 and 2. A significant (P<0.05) increase in level of serum AST, ALT and ALP was found due to CCl₄ treatment in group -II. Whereas in group -III (CCl₄ + extract) rats showed a significant (P<0.05) decrease in AST, ALT and ALP when compared to group -II. This is comparable with group -IV (Silymarin). There was significant increase (P<0.05) in the γ-glutaryl transpeptidase in group- II (CCl₄) as compared to Group-I (Normal control). As significant decrease (P<0.05) was found in group III (CCl₄ + extract) as compared to group II and it was comparable with group- IV and group- I. There was marked decreased in GSH of group- II when compared to group- I. The GSH level significantly increased (P<0.05) in group- III when compared to group- II. There was a significant increase in lipid peroxidase in group- II. Group- III showed marked decrease in lipid peroxidase and it was comparable with Group- I and Group- IV.

**Histopathological Examination**

In control animals, liver sections showed normal hepatic cells with well preserved cytoplasm, nucleus and nucleolus and central vein (Figure 1). In CCl₄ treated animals, the sections showed fatty changes in centrilobular necrosis, steatosis and fatty vaculization were seen with acute inflammatory cells infiltration sinusoids mainly in central zone (Figure 2). In CCl₄ and CV treated animals, the sections showed mild fatty change and mild sinusoidal congestion (Figure 3). In CCl₄ and silymarin treated animals, the sections showed mild sinusoidal congestion and mild central venous congestion (Figure 4).

**DISCUSSION**

The CCl₄ is one of the most commonly used hepatotoxins in experimental study of liver disease (11). The lipid peroxidative degradation of biomembrane is one of the principle causes of hepatotoxicity of CCl₄ (12,13). This is evident from an elevation in the serum marker analysis namely AST, ALT and ALP. *Cleome viscosa* (CV) significantly reduced this serum enzyme in group - III. Simultaneous administration of CV and CCl₄ produced significant recovery of the liver damage induced by CCl₄.

The hepatotoxic effect of CCl₄ are largely due to its active metabolite trichloromethyl radical (14), which binds to the macromolecule and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polysaturated fatty acids. This leads to the formation of lipid peroxide which in turn gives toxic aldehyde that causes damage to liver (15). This was evidenced by increase in level of lipid peroxidation in CCl₄ group and there was significant
Table 1: Effect of Cleome viscosa on serum enzyme in CCl₄ induced hepatic damage in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drugs</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle Control</td>
<td>107.6 ± 9.5</td>
<td>59.5 ± 4.1</td>
<td>249.5 ± 18.2</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄ (0.5ml/kg i.p.)</td>
<td>378.9 ± 23.7ᵃ</td>
<td>254.9 ± 19.3ᵃ</td>
<td>586.9 ± 31.6ᵃ</td>
</tr>
<tr>
<td>III</td>
<td>CCl₄ + CV (200mg/kg p.o.)</td>
<td>117.6 ± 6.7ᵇ</td>
<td>66.2 ± 6.1ᵇ</td>
<td>258.4 ± 16.2ᵇ</td>
</tr>
<tr>
<td>IV</td>
<td>CCl₄ + Silymarin (100mg/kg p.o.)</td>
<td>111.7 ± 8.7ᵇ</td>
<td>62.3 ± 5.1ᵇ</td>
<td>255.3 ± 24.1ᵇ</td>
</tr>
</tbody>
</table>

Values are in mean ± SEM. Number of animals in each group = 6; ᵃ P<0.05 Vs group I. ᵇ P<0.05 Vs group II

Table 2: Effect of CleomeViscosa on Liver γ-glutamyl transpeptidase, Glutathiones and Lipid Peroxidase in CCl₄ induced hepatic damage in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drugs</th>
<th>γ-Glutamyl transpeptidase µmol/mg tissue</th>
<th>Glutathione mg/gm tissue</th>
<th>Lipid peroxide Mol/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle Control</td>
<td>44.06 ± 5.1</td>
<td>46.8 ± 4.8</td>
<td>136.9 ± 11.1</td>
</tr>
<tr>
<td>II</td>
<td>CCl₄ (0.5ml/kg i.p.)</td>
<td>198.8 ± 13.8ᵃ</td>
<td>20.9 ± 2.1ᵃ</td>
<td>289.5 ± 18.2ᵃ</td>
</tr>
<tr>
<td>III</td>
<td>CCl₄ + CV (200mg/kg p.o.)</td>
<td>59.5 ± 4.8ᵇ</td>
<td>35.6 ± 4.6ᵇ</td>
<td>165.5 ± 15.6ᵇ</td>
</tr>
<tr>
<td>IV</td>
<td>CCl₄ + Silymarin (100mg/kg p.o.)</td>
<td>46.7 ± 3.2ᵇ</td>
<td>41.3 ± 4.1ᵇ</td>
<td>155.5 ± 11.5ᵇ</td>
</tr>
</tbody>
</table>

Values are in mean ± SEM : Number of animals in each group = 6 ; ᵃ P<0.05 Vs group I. ᵇ P<0.05 Vs group II

Figure 1: Liver tissue of control rats showing normal histology

Figure 2: Liver tissue of CCl₄ treated rats showing centrilobular necrosis, steatosis and fatty vacuolaization were seen with acute inflammatory cells infiltration sinusoids mainly in central zone

Figure 3: Liver tissue of rats with CCl₄ and CV treated animals, the sections showed mild fatty change and mild sinusoidal congestion

Figure 4: Liver tissue of rats with CCl₄ and silymarin the sections showed mild sinusoidal congestion and mild central venous congestion
decrease in lipid peroxidation in CCl₄ and CV treated groups.

The estimation of γ-GTP level is valuable screening test with high negative, predictive nature for liver disease (16). In present study γ-GTP activity was elevated in CCl₄ treated rats. Increased activity of γ-GTP indicate a severe damage to tissue membrane during CCl₄ toxicity, because of γ-GTP which is a membrane bound enzyme (17). Administration of CV with CCl₄ to rats showed reduction in γ-GTP activity thus, it could indicate and reflect the membrane stabilizing activity of CV. This indicated that CV resulted in improving in liver function.

GSH plays a protective role in tissue by detoxification of xenobiotics. The tripeptide reduced GSH is essential to maintain structural and functional integrity of the cell. The significant decrease in liver GSH in CCl₄ treated rats may be due to enhanced substrate utilization by glutathione peroxidase (18). Administration of CV during severe liver damage condition has elevated GSH levels, which is turn helps in maintaining the liver tissue damage.

Comparative histopathological study of the liver from different groups of rats corroborated the hepatoprotective efficacy of CV. various pathological changes like steatosis, centrilobular necrosis and vacuolization seen in group II rats were prevented to a moderate extent in groups III and IV. All the effects of CV were comparable with silymarin. The results of our study indicated that the aqueous seed extract of Cleome viscosa could protect liver against CCl₄ induced hepatotoxicity.

REFERENCES
PHCOG MAG.: Research Article

In-vitro antioxidant activity of an Adaptogenic Homeopathic formulation

Ashvin V.D. and Mishra S.H*

Department of Pharmacy, M.S.University of Baroda, Vadodara-390002, Gujarat, India.

*Correspondence- shmishra48@rediffmail.com

ABSTRACT - The Homeopathic system of medicine relies mainly on plants and minerals as drug components. There are several herbo-mineral formulations available, which were claimed for their potential as tonic. One of such Homeopathic formulations containing adaptogenic plants, as ingredients, was prepared in the laboratory. The laboratory formulation was subjected to evaluation of in vitro antioxidant potential and compared with that of the marketed formulation. The formulations as well as the individual components exhibited antioxidant properties. The total phenolic content of the formulation and of the individual components were determined and correlated with the antioxidant activities. The present study showed that the Homeopathic medicines, administered even in much diluted form; exhibit significant in-vitro activities, which also justify the principle of Homeopathy Doctrine.

KEY WORDS: Adaptogenic, Homeopathic system of medicine, In-vitro Anti-oxidant activity, Tonic.

INTRODUCTION

Homeopathy is one of the alternative systems of medicine (1) having a well-documented pharmacopoeia. The monograph of pharmacopoeia includes generally the information on individual substances used as medicament. It has become common practice, now in homeopathic system, similar to modern system of medicine, of providing a multi component dosage forms. These dosage forms contain tinctures of herbal products and minerals either in combination or in singular form.

There is paradox that the medicinal power of substance increases as its quantity decreases - even to the point of being physically absent from the solution (2). Hahnemann believed that the process of dilution and succussion or trituration actually released a ‘spirit-like’ healing power that is particularly adapted to work on the equally spirit-like vital force in people (3).

The homeopathic system of medicine relies mainly on plant and mineral components as drugs. There are several homeopathic herbo mineral formulations available, which are used as tonic. Tonics are preparations, which mitigate the conditions of weakness or lack of tone within the entire organism, or in particular organ (4). Tonic effect thus, can be considered, as a part of the adaptogenic potential of the formulation. Adaptogenic potential of the drug may be attributed to its modulation effect on the functions of the various physiological systems of the body (5). The word Adaptogens was, first defined in the 1950 by Lazarev, as substances that normalize body functions, strengthen systems and functions compromised by stress and have a protective effect against a wide variety of environmental and emotional stress (6). Adaptogens are characterized by their anti-stress effects towards stresses of a non-infectious variety. Thus, the experiments were designed to access the adaptogenic properties of the selected tonic formulation and individual components from homeopathic system of medicine by determining their in vitro antioxidant properties. One such marketed formulation (7), was also evaluated in similar manner. Homeopathic formulation contain very diluted forms of plant mother tinctures, may be up to $10^{-1}$ X concentration power and some mother tinctures of minerals which is present in dilution of $10^{-6}$ X power in a preparation. These are prepared by using special method and in absolute alcohol, which is known as trituration and succussion in homeopathic formulary language. Phenolic compound present in the plant mother tincture have been considered to be responsible for antioxidant properties in vitro (8, 9).

The total phenolic contents of the formulations as well as of the individual components, thus, were also determined. In the present study an attempt, has been made to a set certain parameters of identification and evaluation of mother tinctures of individual plant drugs as well as their formulation although in very diluted form, but shows significant activity supporting the
principle of Homeopathic medicine “As the concentration decreases the potency or activity of drug is increases”, when subjected to evaluate certain parameters of evaluation and identification.

**MATERIALS AND METHODS**

**Materials**

The marketed homeopathic Alfalfa tonic selected for present study was labeled to contains diluted forms of Mother Tincture each of Alfalfa (Medicago sativa) (10), Cinchona officinalis (11), Avena sativa (12), Hydrastis Canadensis (13), Withania Somnifera (14), claimed with adaptogenic properties. The plant materials, of which tinctures were used in formulation, were individually procured from the local market. The samples of plant materials were identified by comparing them with herbarium specimens preserved in the Botany Department of M.S. University of Baroda. Tincture of Hydrastis was directly obtained from a local market. All reagents used, were of, pure grade, unless mentioned, otherwise in the description.

**Preparation of Mother Tinctures**

The Mother Tinctures of each of the procured drug sample, were prepared in the laboratory, using the given procedure, and then subjected to dilution in different grades as prescribed in Homeopathic Formulary (15, 16). Alfalfa mother tincture was prepared by using fresh plant leaves of Medicago sativa, cut into small pieces and crushed in mortar and pastle. Afresh juice was obtained from the crushed leaves of Alfalfa by expression method using a new linen cloth. The juice obtained was weighed and equal quantity by weight of alcohol was added to it. The mixture was shaken vigorously for sometimes then allowed to stand in cool and dry place for eight days. The tincture was filtered, and stored in glass bottle provided with non-porous velvet cork. This tincture is termed as mother tincture in Formulary of Homeopathy. 2 minims of this mother tincture was added to 8 minim of dilute alcohol and then the mixture was given 10 downward stroke of equal strength (succession), which gave 1X potency preparation. All succeeding potencies may then be prepared by taking 1 minim of the preceding potency and 9 minim of dilute alcohol.

Cinchona tincture was prepared by using weighed quantity of fine powder of dried bark of Cinchona officinalis and five times of its weight alcohol was mixed in a glass jar. After mixing the whole mass was kept in cool and dark place for 15 days. The clear tincture was decanted; the residue was strained by new linen cloth and was added to the previously decanted tincture. The filtered tincture was stored in glass stoppered bottle with non-porous velvet cork. The succeeding potencies may be prepared similarly as stated for Alfalfa tincture.

Withania tincture was prepared by using fresh roots of Withania Somnifera which were cut in to small pieces and crushed in mortar and pestle. Every three parts of weighed roots were treated with two parts by weight of alcohol and allowed to macerate for five days. The remaining procedure followed was same as mentioned for cinchona mother tincture.

Avena tincture was prepared using pulverized seeds of Avena sativa. A weighed quantity was taken and to it double the quantity by weight alcohol was added. The content was allowed to macerate for eight days, after this period the tincture is decanted, and filtered through a linen cloth and, stored, 6 minim of this mother tincture are mixed with 8 minim of dilute alcohol and the mixture was given 10 downward stroke of equal strength (succession) to give 1X potency preparation. All succeeding potencies may be prepared by taking 1 minim of preceding potency and 9 minim of dilute alcohol.

Calcium Phosphate mother tincture was prepared by triturating with one part by weight of calcium phosphate with 99 parts by weight of sugar of milk (Lactose), which gave first triturate. The second triturate was prepared by taking one grain of first triturate with 99 grains of sugar of milk. The third triturate was prepared by triturating 1 minim of second triturate with 99 grains of sugar of milk. One grain of third triturate was dissolved in 50 minim of purified water and mixed with 50 minim of alcohol yielded 4th potency. All succeeding potencies were prepared by mixing 1 minim of preceding potency and 9 minim of dilute alcohol and giving it 10 downward strokes. Similar methods were adopted to prepare Ferrous phosphate tincture, Sodium phosphate tincture, Potassium phosphate tincture.

**Preparation of formulation**

A liquid formulation was prepared in the laboratory based on the labeled contents of a marketed homeopathic herbal formulation. The ingredients were incorporated in the following manner. Each 5ml tonic contains - Alfalfa tincture of 2X potency 0.325 ml, Cinchona officinalis tincture of 3X potency 0.0075 ml, Withania Somnifera tincture of 2X potency 0.0175 ml, Avena sativa tincture of 3X potency 0.0175 ml, Hydrastis canadensis tincture of 2X potency 0.0175 ml, acid phosphate tincture of 2X potency 0.0175 ml, Ferrous phosphate tincture of 6th potency 0.25 ml,
Sodium phosphate tincture of 6th potency 0.025 ml, Magnesium phosphate tincture of 6th potency 0.025 ml, Potassium phosphate tincture of 6th potency 0.025 ml, Calcium phosphate tincture of 6th potency 0.025 ml. The laboratory formulation was prepared by mixing one after another, the tinctures of different potency of specified quantity of each ingredient and finally the volume was made up with distilled water. The formulation so obtained was then used for further investigation on following lines.

Preparation of sample for analysis
The tinctures of Withania somnifera, Avena sativa, Cinchona officinalis, Hydrastis Canadensis, Medicago sativa (Alfalfa), Laboratory preparation and Market preparation were dried to obtain solid mass at control temperature not to exceed 50°C first on a water bath and then subjected to vacuum drying. The solid mass was then dissolved in methanol to obtain a uniform concentration of 1 mg/ml. These samples were used for further studies in following manner.

Total Phenolic content
Total phenolic content was determined using Folin - Ciocalteau method. Each of the 100μl of samples of tinctures as well as laboratory and market preparation was taken in to 25ml volumetric flask, to which 10ml of water and 1.5ml of Folin Ciocalteau reagent were added. The mixture was then kept for 5 min. and to it 4ml of 20% w/v sodium carbonate solution was added the volume was made up to 25ml with double distilled water. The mixture was kept for 30 minute until blue color develops. The samples were then observed at 765 nm in UV-visible spectrometer Shimadzu, UV-1601, Japan. The % of total phenolic was calculated from calibration curve of Gallic acid plotted by using similar procedure (17-19).

The DPPH free radical scavenging activity, Super oxide free radical scavenging activity and Nitric oxide scavenging activity were calculated using the following formula:

% Reduction = \( \frac{\text{Control absorbance} - \text{Test absorbance}}{\text{Control absorbance}} \times 100 \)

The result of Total Phenolic content, DPPH free radical scavenging activity, Super oxide free radical scavenging activity, Nitric oxide scavenging activity were compared for Laboratory and Marketed preparation by using paired t-test.

DPPH free radical scavenging activity
4.3mg of DPPH (1, 1-Diphenyl -2-picrylhydrazyl) was dissolved in 3.3 ml methanol; it was protected from light by covering the test tubes with aluminum foil. 150μl DPPH solution was added to 3ml methanol and absorbance was taken immediately at 516nm for control reading. Different volumes, of samples of mother tinctures of different drug as well as laboratory and market preparation, measuring 50μl, 60μl, 70μl, were taken and the volume was made uniformly to 150μl using methanol. Each of the samples was then further diluted with methanol up to 3ml and to each 150μl DPPH was added. Absorbance was taken after 15 min. at 516nm using methanol as blank on UV-visible spectrometer Shimadzu, UV-1601, Japan. IC50 value for each mother tinctures as well as laboratory and market preparation were calculated (20, 21).

Super Oxide free radical scavenging activity
100μl Riboflavin solution [20 μg], 200μl EDTA solution [12mM], 200μl methanol and 100μl NBT (Nitro-blue tetrazolium) solution [0.1mg] were mixed in test tube and reaction mixture was diluted up to 3ml with phosphate buffer [50mM] The absorbance of solution was measured at 590nm using phosphate buffer as blank after illumination for 5min. This is taken as control. Different volumes of 50μl, 60μl, 70μl of samples of each of mother tinctures of different drugs as well as laboratory and market preparation, were taken and diluted up to 100μl with methanol, to each of this, 100μl Riboflavin, 200μl EDTA, 200μl methanol and 100μl NBT was mixed in test tubes and further diluted up to 3ml with phosphate buffer. Absorbance was measured after illumination for 5min. at 590nm on UV visible spectrometer Shimadzu, UV-1601, Japan. Similar procedure is followed for other tinctures and laboratory and market preparation and adjusting the test sample volume 100 μl. IC50 value for each mother tinctures as well as laboratory and market preparation were calculated (22, 23).

Nitric Oxide scavenging activity (24-26)
50μl, 60μl, 70μl, of each of the samples of mother tinctures of different drugs as well as laboratory and market preparation were taken in separate tubes and the volume was uniformly made up to 150μl with methanol to each tube 2.0 ml, of sodium nitroprusside (10 mM) in phosphate buffer saline was added. The solutions were incubated at room temperature for 150 minutes. The similar procedure was repeated with methanol as blank which served as control. After the incubation, 5 ml of Griess reagent was added to each tube including control. The absorbance of chromophore formed was measured at 546 nm on UV-visible spectrometer Shimadzu, UV-1601, Japan. Curcumin was used as positive control IC50 value for each mother tinctures as well as laboratory and market preparation were calculated.
RESULTS
The test for total phenolic content was carried out on individual tinctures as well on laboratory preparation and on market formulation. It was observed that tincture of cinchona had highest phenolic content then rest of the tinctures. The phenolic content of marketed and laboratory preparation were quite comparable as showed in Table-1. In the DPPH Free radical scavenging activity, the withania tincture showed very potent activity then rest of the tinctures, while the market and laboratory preparation showed potent to moderate activity as mentioned in Table-2. The Super Oxide free radical scavenging activity was found highest in hydrastis tincture while rest of tinctures and market as well as laboratory preparation showed significant activity as in Table-3. Among the tinctures studied, nitric oxide scavenging activity was found only in Withania and Hydrastis while moderate activity was also observed in market and laboratory preparation as mentioned in Table-4. The result of Total Phenolic content, DPPH free radical scavenging activity, Super oxide free radical scavenging activity, Nitric oxide scavenging activity were compared for Laboratory and Marketed preparation by using paired t-test and results were found to be significant for DPPH free radical activity and Nitric oxide activity while results of Total Phenolic content and Super oxide free radical scavenging activity were found extremely significant.

Table-1: Total Phenolic content

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Phenolic in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Withania somnifera</td>
<td>416.10 ± 0.51</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>422.47 ± 1.19</td>
</tr>
<tr>
<td>Cinchona officinalis</td>
<td>746.8 ± 2.19</td>
</tr>
<tr>
<td>Hydrastis canadensis</td>
<td>421.67 ± 2.21</td>
</tr>
<tr>
<td>Medicago sativa (Alfalfa)</td>
<td>651.03 ± 1.51</td>
</tr>
<tr>
<td>Laboratory Preparation</td>
<td>587.67 ± 1.82</td>
</tr>
<tr>
<td>Market Preparation</td>
<td>605.80 ± 1.36</td>
</tr>
</tbody>
</table>

* The value is expressed as µg of Gallic acid equivalent / ml of sample
* The regression values and correlation of regression of Gallic acid were
  \[ y = 0.0042x + 0.0187 \] and \[ R^2 = 0.9916 \]
* The Market preparation and laboratory were compared by using paired t-test and t value found was 68.265 and p value is 0.002 which was considered extremely significant.

Table-2: DPPH Free radical scavenging activity

<table>
<thead>
<tr>
<th>Dose</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; in µg/ml</th>
<th>Regression equation</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (Standard)</td>
<td>18.53 ± 0.51</td>
<td>( y = 1.786x - 0.009 )</td>
<td>0.9996</td>
</tr>
<tr>
<td>Withania somnifera</td>
<td>10.87 ± 1.17</td>
<td>( y = 1.1691x + 45.06 )</td>
<td>0.9923</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>57.92 ± 1.17</td>
<td>( y = 2.6109x - 101.21 )</td>
<td>0.9991</td>
</tr>
<tr>
<td>Cinchona officinalis</td>
<td>12.19 ± 1.02</td>
<td>( y = 1.5364x + 31.27 )</td>
<td>0.9788</td>
</tr>
<tr>
<td>Hydrastis canadensis</td>
<td>17.80 ± 1.91</td>
<td>( y = 0.7301x + 36.99 )</td>
<td>0.9981</td>
</tr>
<tr>
<td>Medicago sativa (Alfalfa)</td>
<td>63.30 ± 1.91</td>
<td>( y = 0.8974x - 5.67 )</td>
<td>0.9949</td>
</tr>
<tr>
<td>Laboratory Preparation</td>
<td>64.45 ± 1.07</td>
<td>( y = 1.9151x - 73.62 )</td>
<td>0.9987</td>
</tr>
<tr>
<td>Market Preparation</td>
<td>61.32 ± 2.19</td>
<td>( y = 0.6935x + 8.86 )</td>
<td>0.9975</td>
</tr>
</tbody>
</table>

* The Regression values and correlation of regression of Gallic acid were
  \[ y = 0.0042x + 0.0187 \] and \[ R^2 = 0.9916 \]

Table-3: Super Oxide free radical scavenging activity

<table>
<thead>
<tr>
<th>Dose</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; in µg/ml</th>
<th>Regression equation</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (Standard)</td>
<td>18.16 ± 2.19</td>
<td>( y = 1.345x + 1.23 )</td>
<td>0.9998</td>
</tr>
<tr>
<td>Withania somnifera</td>
<td>50.74 ± 2.37</td>
<td>( y = 0.9854x - 0.0054 )</td>
<td>0.9976</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>68.38 ± 1.96</td>
<td>( y = 1.0809x - 19.592 )</td>
<td>0.9966</td>
</tr>
<tr>
<td>Cinchona officinalis</td>
<td>66.70 ± 0.57</td>
<td>( y = 1.079x - 21.62 )</td>
<td>0.9955</td>
</tr>
<tr>
<td>Hydrastis canadensis</td>
<td>28.63 ± 1.27</td>
<td>( y = 1.19x + 18.61 )</td>
<td>0.9989</td>
</tr>
<tr>
<td>Medicago sativa (Alfalfa)</td>
<td>48.68 ± 2.37</td>
<td>( y = 0.7529x + 18.61 )</td>
<td>0.9983</td>
</tr>
<tr>
<td>Laboratory Preparation</td>
<td>61.29 ± 2.19</td>
<td>( y = 1.2055x - 23.885 )</td>
<td>0.9976</td>
</tr>
<tr>
<td>Market Preparation</td>
<td>66.71 ± 1.67</td>
<td>( y = 0.7082x + 2.759 )</td>
<td>0.9993</td>
</tr>
</tbody>
</table>

* The Regression values and correlation of regression of Gallic acid were
  \[ y = 0.0042x + 0.0187 \] and \[ R^2 = 0.9916 \]

* The Market preparation and laboratory were compared by using paired t-test and t value found was 68.265 and p value is 0.002 which was considered extremely significant.
Table-4: Nitric Oxide scavenging activity

<table>
<thead>
<tr>
<th>Dose</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; in µg/ml</th>
<th>Regression equation</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>10.52 ± 1.72</td>
<td>y = 1.234x – 8.112</td>
<td>0.9996</td>
</tr>
<tr>
<td>(Standard)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Withania</td>
<td>48.97 ± 0.87</td>
<td>y = 0.9105x + 5.415</td>
<td>0.9976</td>
</tr>
<tr>
<td>somnifera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avena sativa</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinchona officinalis</td>
<td>- -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrastis canadensis</td>
<td>50.83 ± 1.84</td>
<td>y = 0.6331x</td>
<td>0.9991</td>
</tr>
<tr>
<td>Medicago sativa (Alfalfa)</td>
<td>1.84 + 17.817</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory</td>
<td>69.45 ± 1.23</td>
<td>y = 0.5378x + 18.171</td>
<td>0.9989</td>
</tr>
<tr>
<td>Preparation</td>
<td>67.47 ± 2.16</td>
<td>y = 0.4626x</td>
<td>0.9994</td>
</tr>
</tbody>
</table>

* The sign R<sup>2</sup> is correlation of Regression.
* The Market preparation and laboratory were compared by using paired t-test and t value found was 4.563 and p value is 0.0448 which was considered significant.

DISCUSSION

Traditional medicament play an important role in our day to day life in spite of overwhelming influence of modern medicine in treatment of various disorders like diabetes, viral infection, rheumatic disease, allergic condition, obesity, respiratory diseases, cardiovascular diseases, etc. Although number of poly herbal formulations are used in traditional system but only a few are accepted in modern medicine due to lack of accurate method for their standardization and evaluation.

Homeopathic dosage forms are widely used, containing tinctures of herbal products and minerals either in combination or in singularly. In general, scientifically acceptable methods for standardization of these preparations are not available. The present studies were planned to evolve certain parameters, and generate some data for evaluation and standardization of a selected poly herbal homeopathic adaptogenic tonic from the market. A similar product was prepared in the laboratory and both were subjected for investigation. The mother tinctures used in the preparation were found rich in phenolic content and so also the laboratory and market preparation, even though these contain diluted quantities their anti oxidant activity can therefore be correlated with phenolic content.

The DPPH scavenging activity, super oxide scavenging activity and nitric oxide scavenging activity exhibited by the samples were comparable with reference compound, ascorbic acid in first two and Curcumin for the third activities respectively. The IC<sub>50</sub> value of Withania tincture, Hydrastis mother tincture, and Cinchona mother tincture shows higher antioxidant activity. The Alfalfa mother tincture and Avena mother tincture have moderate antioxidant activity. The laboratory preparation and market preparation show moderate activity even though they contain lower concentration of these tinctures, may be due to some synergic action which support the principle of Homeopathy. On the whole, the studies offer impetus to the need of evaluation of marketed preparation of Homeopathic system in order to generate confidence, both among the practitioners and patients.

REFERENCES

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