IJBPAS, June, 2012, 1(5): 684-695

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

EVALUATION OF SAFETY PROFILE OF *ZANTOXYLUM LIMONELLA* FRUIT

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ABSTRACT

The use of medicinal plants as raw materials in the production of new drugs is ever increasing because of their potentials in combating the problem of drug resistance in micro-organisms. Demand for medicinal plants is increasing in both developing and developed countries. Research on medicinal plants is one of the leading areas of research globally. However, there is a need to pay closer attention to the issue of bioactivity-safety evaluation and conservation of medicinal plants. According to WHO guide lines, a herbal drug needs to be standardized with respect to safety before it is used for making the formulation. In the present studies, source safety evaluation parameters were considered. The experiment was conducted on Kerala sample, Mumbai sample and Delhi sample. The average values were recorded.

Keywords: Herbal Drugs, Safety Parameter, Herbal Drug Toxicity

INTRODUCTION

Medicinal plants are plants containing inherent active ingredients used to cure disease or relieve pain **[1]**. The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed **[2]**. The World Health Organization estimated that 80% of the population's of developing countries rely on traditional medicines, mostly plant drugs, for their primary health care needs **[3]**. Herbs have created in interest among the people by its clinically proven effects like immunomodulation, adaptogenic and anti mutagenic etc. Also, the medicinal properties of plants could be based on the antioxidant, antimicrobial antipyretic effects of the phytochemicals in them **[4, 5]**.

Injuries to the liver associated with marked alteration in liver chemistry have been treated at various times using crude extracts of plants **[6, 7]**. In the absence of reliable liver protective drugs in allopathic medical practices, herbs play an important role in the management of various liver disorders **[8]**. A number of plants haveshown hepatoprotective property **[9, 10]**. Traditionally, herbs have been considered to be nontoxic and have been used for treating various problems by the general public ‘‘and/or’’ traditional medicine doctors worldwide **[11]**. Herbal remedies are plant-based, as in fact are many orthodox drugs - some 25% of present pharmaceutical preparations contain at least one active ingredient extracted from plant sources **[12]**. Moreover, thousands of our present drugs were originally derived from plants, including digitalis (foxglove), aspirin (willow and meadowsweet) and paclitaxel (Taxol).However, in the case of orthodox drugs the active ingredient is isolated from the plant, chemically standardized, subjected to critical clinical assessment and then often replaced with a synthetic analogue. Although, the literature has documented several toxicity resulting from the use of herbs on many occasions, still the potential toxicity of herbs has not been recognized by the general public or by professional groups of traditional medicine **[13, 14]**. Herbal medicines are not required to undergo the same quality checks as conventional drugs and so they may be contaminated or adulterated. For example, many Asian and Indian herbal remedies have been found to contain heavy metals such as lead, arsenic and mercury. One of the worst examples of contamination causing adverse effects occurred in the USA in 1989, where there was an outbreak of eosinophilia-myalgia syndrome associated with the use of Ltryptophan,

An over-the-counter dietary supplement used for weight loss **[15]**. More than 1500 cases were reported, including 38 deaths. More than 95% of the cases were traced to an individual Japanese supplier. Researchers found some trace-level impurities, suggesting that a contaminated batch of L-tryptophan contributed to the outbreak. The use of medicinal plants as raw materials in the production of drugs is again gaining popularity and the need for effective conservative strategies for medicinal plants **[16]**.

The objective of this study is to emphasize the need of a closer attention towards the vital issues of safety evaluation and conservation of medicinal plant *Zantoxylum Limonella* due to increase in their use as potential alternatives and as cheaper and safer alternatives to chemical drugs.

Frequent Contaminants in Plant Drugs

Aflatoxins

Aflatoxins are naturally occurring mycotoxins that are produced by many species of Aspergillus, a fungus, the most notable ones being Aspergillus flavus and Aspergillus parasiticus. Their name is derived from the early work that discovered *Aspergillus flavus* toxins. Aflatoxins are toxic and among the most carcinogenic substances known **[17]**. After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful aflatoxin M1. Aflatoxin-producing members of Aspergillus are common and widespread in nature. They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by Aspergillus following prolonged exposure to a high-humidity environment, or damage from stressful conditions such as drought, a condition that lowers the barrier to entry.

Children, however, are particularly affected by aflatoxin exposure, which leads to stunted growth and delayed development **[18]**. Chronic exposure also leads to a high risk of developing liver cancer, as aflatoxin metabolite can intercalate into DNA and alkylate the bases through its epoxide moiety. This is thought to cause mutations in the p53 gene, an important gene in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis. These mutations seem to affect some base pair locations more than others — for example, the third base of codon 249 of the p53 gene appears to be more susceptible to aflatoxin-mediated mutations than nearby bases **[19]**.

Pesticide Residue

Pesticide residue refers to the pesticides that may remain on or in food after they are applied to food crops **[19]**. The levels of these residues in foods are often stipulated by regulatory bodies in many countries. Many of these chemical residues, especially derivatives of chlorinated pesticides, exhibit bioaccumulation which could build up to harmful levels in the body as well as in the environment **[20]**. Many pesticides achieve their intended use of killing pests, by disrupting the nervous system. Due to similarities in brain biochemistry among many different organisms, there is much speculation that these chemicals can have a negative impact on humans as well **[21]**.

Microbial Contamination

Microbial contamination frequently involves in herbal products since all products come from plants. Therefore, microbial contents in herbal products should be evaluated. Microbial contamination of herbal medicines can be influenced by

environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the storage conditions of crude and processed medicinal-plant materials. For these reasons, there is currently a global danger to the health and well-being of the people. The World Health Organization, British Pharmacopoeia and the United States Pharmacopoeia have recommended tolerable microbial limits in non-sterile pharmaceutical products which include 107 cfu/ml bacteria and 105 cfu/ml fungi

Mineral Contamination

The use of herbal and traditional medicine leads a misconception that natural means safe **[22]**.The contamination of herbal remedies with heavy metals due to soil and atmospheric contamination poses athreat to its safety. Health implication of heavy metal poisoning are abound in many literature **[23]**.

MATERIALS AND METHODS

Determination of Minerals

The powdered sample was cleaned manually and there were no dust particle. The sample was dried at 150 degree c to constant weight. The dried sample was used for dry ashing. Pre cleaned silica crucible was heated at 600 degree until the weight of the crucible was constant. About 5gm sample was taken in the silica and heated in muffle furnace at 400deree till there was no evolution of smoke. The crucible was then taken out, cooled at room temperature by keep in it in a desecrator and the ash was moistened with concentrated sulphuric acid (0.5ml). It was then heated on a heating mental till the fumes of sulphuric acid ceased. The crucible with sulphated ash was then heated in muffle furnace at 600 degree till the weight of the content was constant (2 to 3hrs) **[24]**.

The sulphated ash obtained above was then dissolved in 100ml of 5% hydrochloric acid solution (5ml conc. Hydrochloric acid +95ml double distilled water).The solution was stored in tightly capped plastic bottles and directly used for the determination of various mineral elements bu usin atomic absorption spectrophotometer, model, shimadzu, AA-640-13 **[25]**.

Standard solutions of all the mineral elements were prepared, the instrument was calibrated and the concentration of minerals in ppm was measured directly in the yest solution **[26]**.

Microbial Count

Determination of microbial count was conducted according to WHO guidelines **[27, 28]**.

Qualitative Detection of Aflatoxins

Sample Preparation

About 50 g of the powdered drug was weighed into a mixing jar, added 25 ml of saturated sodium chloride solution and 250 ml of Methylene chloride. Blended for 3 min at high speed. Filtered through high porosity folded paper into 50 ml graduated cylinder. Transferred 50, l filtrate to 250 ml glass stoppered Erlenmeyer flask. Evaporated the extract to near dryness on staem bath and added methanol: 5% sodium hydroxide: hexane (50: 50: 50) ; shaken for10 min's on wrist action shaker, and then transferred to 250 ml separating funnel. Allowed to stand for 5–10 mins, drained lower aqueous layer into another 250 ml separating funnel. Added 50 ml of carbon tetra chloride to aqueous layers, shaken vigorously for 1 min and allowed to separate the layer. Added 50 ml of methylene chloride to retain aqueous layer, shaken for 1 min, drined methylene chloride layer into 250 ml Erlenmeyer flask and extracted aqueous layer with additional 25 ml methylene chloride. Combined the methylene chlodide extracts, evaporated the combined methylene chloride extracts to near dryness on steam bath **[29]**.

Column Chromatography

Place a bed of glass wool in the bottom of chromatographic column and added 1cm high anhydrous sodium sulphate to give base for silica gel. Added methylene chloride to settle silica, there was 3cm high methylene chloride. Slowly added 2cm bed of anhydrous sodium sulphate **[30]**.

Dissolved the extract in 5ml methylen chloride ,charged the extract solution to the column. Eluted sequentially at maximum flow rate with 40 ml methylene chloride, 40ml benzene – acetic acid (9:1), 40 ml hexane and 40 ml anhydrous ether, discarded the elutes. Chloroform – acetone (80:20) was used as an eluting system for aflatoxins. Elute was collected and evaporated to dryness on steam bath under nitrogen. Reserved this dry extract for thin layer chromatography **[31]**.

Aflatoxins Reference Sample

Different Aflatoxin reference samples were prepared in Benzene-Acetonitrile (98:2).

Particulars of TLC

Thin layer plates: Percolated silica gel G F254 plates (10x20cm) of uniform thickness (0.2mm).

Chromatographic chamber: Glass tank with a lid

Solvent system: Chloroform – acetone – isopropanol (85:10:05)

Detection: Under UV chamber fitted with 15 watt long wave ultraviolet lamp

Qualitative Detection of Pesticide Residues

Test Sample

20 – 50 gram of the powder was taken, added acetonitrile – water mixture (650:350) and blended for 5 min at speed and filtered. Transferred the filtrate into one liter separating funnel and added 100 ml of light petroleum ether. The contents were shaken for one to two minutes and added 10 ml of sodium chloride (400 g / litre) and 600 ml of distilled water. Shaken the separating funnel vigorously for 30 – 40 seconds and allowed the solvent layer to get separated. Collected the petroleum ether layer , washed with water (thrice). Then treated with anhydrous sodium sulphate. The extract was subjected to column chromatography, The column was packed with activated florosil and the column was eluted with petroleum ether. Collected three fractions of 200 ml each **[32]**.

The first elute contains chlorinated pesticides like aldrein, benzene hexa chloride, DDt, etc. While second elute contains dieldrein, the third contains malathion **[33]**. The elutes were concentrated to 10 ml and was used for the thin layer chromatography **[34]**.

Standard samples: all the reference samples were prepared in petroleum ether.

Adsorbent: Precoated silica gel G F_{254} plate (10 x 20 cm) of uniform thickness (0.2 mm)

Solvent system: n – hexane:Acetone (7:3)

Detection: 1. under iodine treatment

2. under UV chamber.

RESULTS AND DISCUSSION

Earlier Study showed that more than 98% of the Herbal studies on herbal medicines are well documented in Remedies (HR) analyzed did not comply with literature. Percentage concentration of various elements in *Zanthoxylum limonella* is given at **Table 1**. The present study reports microbial contaminations **(Table 2)** in herbal products widely distributed over the country. So the experiment was performed to investigate microbial contamination of collected samples from different places (like Kerala, Maharastra and Delhi) against microorganisms representing human pathogens **(Table 3-4)**. Preservatives may be added to some herbal products during the

manufacturing process or the product itself may retain the inhibitory effect on microorganisms. Either one of this problem would have an effect on microbial determination of the product. It was shown that all samples have very low or no microbial contamination. The Pharmacopoeia recommends that the total aerobic count for non-sterile edible products should not be over 5.0 x 105 colonies per gram or millilitre of the sample and the total yeasts and molds count should not be more than 5.0 x 103 colonies per gram of the sample. Surprisingly, *Zanthoxylem limonella* samples did almost meet the microbiological criteria in the Pharmacopoeia. Furthermore, the level of alfa toxin and the residue of pesticide also found nill in Z. *lomonella* fruits **(Table 5 & 6)**.

Table-1: Percentage Concentration of Elements in *Zanthoxylum limonella*

S. No.	Name of the Element	% Concentration (ppm) (n = 3)
01.	Calcium	0.52
02.	Magnesium	0.41
03	Aluminium	0.082
04	Arsenic	Nil
05	Cadmium	Nil
06	Zinc	0.07
07	Lead	Nil
08	Chromium	Nil
09	Mercury	Nil

n = average value of three samples

Table 2: Tests for Microbial Contamination in *Zanthixylum limonella*

Organism	Colony Characteristics	Biochemical Test			Probable no: of organism/ gm of sample. (n= 3)
		Test	+ ve control	- ve control	
Salmonella typhi	**Small transparent ,and colorless, opaque pink (surrounded by a pink to red zone)indicates the presence of *s. typhi***	**Absent**	**Present**	**Absent**	**Nil**
Pseudomonas aeruginosa	**Gram – ve rods, usually with a greenish fluorescence indicates the presence of *P. aeruginosa***	**Absent**	**Present**	**Absent**	**Nil**
Staphylococcu	**Black olonies of gram + ve cocci, surrounded by clear zones indicates the presence of**	**Absent**	**Present**	**Absent**	**Nil**

s aureus	*s.aureus*				

Table-3 Test for *E. coli* in *Zanthoxylum limonella*

Organism	Colony Characteristics	Bio chemical test	100 mg/ml	10 mg/ml	0.1m g/ml	0.01mg/m l	Probable no : of Organisms Present / gm of the Sample (n = 3)
E coli	Growth of red, generally non mucoid colonies of gram –ve rods, indicating the presence of *E. Coli*	+ ve	+ve	--	--	--	Less than 10 , more than 1

Table 4: Test for Total Viable Aerobic Bacteria, Yeast and Mould in *Zanthoxylum limonella*

Volume of Stock Solution	No : of tubes in which the microbial growth is seen for each quantity of the preparation being examined	Probable no of organisms/ gm of the sample (n = 3)
0.1 ml (100 mg)	3	1100
0.01 ml (10 mg)	3	1100
0.001 ml (1 mg)	3	1100

Table 5: Qualitative Detection of Aflatoxins in *Zanthoxylum limonella*

Samples	Rf value	Colour	Inference (n= 3)
Aflatoxin B1 (reference)	0.58	Fluorescent blue	The aflatoxins were found absent in the test sample
Aflatoxin B2 (reference)	0.52	Fluorescent blue	
Aflatoxin G1 (reference)	0.47	Fluorescent green	
Aflatoxin G2 (reference)	0.43	Fluorescent green	
Testing sample	Nil	Nil	

Table 6: Qualitative Detection of Pesticide Residues in *Zanthoxylum limonella*

Particulars	Rf value	Observation under		Inference
		Iodine	UV lamp	
Aldrein	0.44	Yellowish light brown	Greenish blue	The test sample was found free from pesticide residues
BHC	0.43	" "	" "	
DDT	0.34	" "	" "	
Dieldrein	0.36	" "	" "	
Malathion	0.43	" "	" "	
Elutes of the testing sample	Nil	___	__	

CONCLUSION

The safety evaluation parameters conducted for *Zanthoxylum limonella* was found to be clear. Three samples collected from Delhi, Mumbai and Kerala were shown the same results. Tests for microbial, pesticide, aflatoxins and heavy metals contamination were proved to be safe and can go for various pharmacological evaluations.

ACKNOWLEDGEMENT

We are extending our sincere thanks to Karpagam University, Coimbatore and Yenepoya Medical, Mangalore, Chairman Rajiv Gandhi trust, Institute of pharmacy to full fill this work.

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