International Journal of Medical Research & Health Sciences

www.ijmrhs.com Volume 2 Issue 1 Jan-Mar 2013 Coden: IJMRHS Copyright @2013 ISSN: 2319-5886

Received: 26th Nov 2012 Revised: 13th Dec 2012 Accepted: 30th Dec 2012

Original research article

PHYTOCHEMICAL SCREENING AND ACUTE TOXICITY STUDY OF ETHANOLIC EXTRACT OF *ALPINIA GALANGA* IN RODENTS.

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ABSTRACT

Introduction: *Alpinia galanga* is an important ingredient in various herbal formulations has reached extensive acceptability as therapeutic agents for several diseases. The investigation of authentic analytical methods, which can reliably profile the phytochemical composition and studies on toxicity profile, including hematological and biochemical parameters is an important initial step for the establishment of standardization to screen further in search of consistent biological activity. **Aim:** To screen ethanolic extract of Alpinia galanga for its phytochemical constituents and acute toxicity profile. **Methods:** Acute toxicity studies done in rodent by OECD guideline 423 and phytochemical analysis by standard laboratory grade reagents. **Results** The present study revealed the presence of complex phytochemical constituents including phenols and flavanoids. The acute toxicity results has classified the test material to fall under the hazard category 2000mg/kg<LD50<5000mg/kg according to globally harmonized classification system. **Conclusion** The present study concludes the safety of ethnobotanical use of *Alpinia galanga* from acute toxicity results and the presence of various phytochemical constituents in *Alpinia galanga* may be responsible for its various pharmacological actions documented in traditional medicine.

Key words: Phytochemical screening, *Alpinia galanga*, Acute toxicity, Ethanolic extract.

INTRODUCTION

The use of medicinal plants and preparations derived from them as dietary supplements, Nutraceuticals, functional foods and herbal medicinal products has become more widely accepted in developing countries. Therefore, it is important to evaluate the adverse effects of these plants and their preparations to increase the confidence in its safety to human, particularly for use in the development of pharmaceutical products. Based on Strobel and Daisy 2003[1] there should be some Rationale for plant selection like plants from unique environmental settings especially those with an unusual biology, plants that have an ethno botanical history, plant that are

endemic that has an unusual longevity and plant growing in areas of great biodiversity. The medicinal plant selected and investigated for present study *Alpinia galanga* has a very strong ethno botanical history endemic in south east Asia and still available as an Ayurvedic preparation for wider therapeutic application including inflammatory disorders. It is used as a constituent of the following Ayurvedic preparations. Rasandi gugluva, Ashvagandaoil, Karpasadi oil, Mahanarayaoil, Mahabalaoil, Vishagananeelaoil, Chyathya ghathya, Vruhathjagaladighathaya, Kakubdhadi powder, Ashvagandadi powder, Ranagigru kvathaya, Ranavishvadiya (Rasna dashamula), Rasna sapthayakaya, Bilvorasnadi kvathaya, Dashamul Iguruadi kvathaya, Dashamuladi kvathaya, Kumaraguliya[2].

Apart from traditional Ayurvedic preparation modern pharmaceutical companies manufacture and provide these preparations in the form of capsules and tablets with standardized packing, few hit products containing *Alpinia galanga* in the formulary includes Rumalaya forte and Orthocare-B for the treatment of arthritis. Based on a review of the literature the present study is an effort to screen phytochemical constituent and study acute toxicity activity of ethanolic extract of *Alpinia galangal*

MATERIALS AND METHODS

Plant Materials

The rhizomes from the plant *Alpinia galanga* were collected from the local areas of Coimbatore (Tamil Nadu, India). The collected material was authenticated by Dr Sasikala Ethirajulu, Assistant Director (Pharmacognosy), Siddha Central Research Institute, Chennai, India.

Experimental animals

Colony inbred animal strains of swiss albino mice of female sex weighing 20-25 g three in each group control and test were used for the toxicological studies according to OECD guidelines. The animals were kept under standard conditions 12:12 (day/night cycles) at 22^0C room temperature, in polypropylene cages. The animals were fed on standard pellet diet and tap water *ad libitum*. The animals were housed for one week in polypropylene cages prior to the experiments to acclimatize to laboratory conditions. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC).

Preparation of Plant Material

The collected rhizome materials were thoroughly washed under running water, shade dried for a week at 35-40^{o}C, pulverized in an electric grinder and exhaustively extracted successively in a Soxhlet apparatus by using the solvent, ethanol. The extracts were concentrated under reduced pressure and were then powdered.

Qualitative Phytochemical Analyses

The phytochemical tests below were carried out on the ethanolic extract of *Alpinia galanga* to determine the active constituents according to the procedures and methods outlined in Barnes, J. (1999) and Harborne[3,4]. These phytochemical tests were done to detect the presence of secondary metabolites, such as alkaloids, tannins, saponins, resins, flavonoids, steroid, glycosides and terpenoids in the plant under investigation.

Test for Alkaloids

A quantity (0.2g) of the sample was boiled with 5ml of 2% HCl on a steam bath. The mixture was filtered and 1ml portions of the filtrate was measured into four test tubes. Each of the 1ml filtrate was treated with 2 drops of the following reagents.

i. **Dragendorff's test:** A red precipitate indicates the presence of alkaloids.
ii. **Mayer's test:** A creamy-white colored precipitate indicates the presence of alkaloids.
iii. **Wagner's test:** A reddish-brown precipitate indicates the presence of alkaloids.
iv. **Picric Acid (1%):** A yellow precipitate indicates the presence of alkaloids.

Test for Flavonoids

A quantity (0.2g) each of the extracts was heated with 10ml of ethyl acetate in boiling water for 3

minutes. The mixture was filtered differently and the filtrates used for the following tests:

i. **Ammonium Test:** A quantity (4ml) each of the filtrates was shaken with 1ml of dilute ammonia solution (1%). The layers were allowed to separate. A yellow coloration was observed at the ammonia layer, which indicates the presence of flavonoids.

ii. **Aluminum Chloride Test:** A quantity (4ml) each of the filtrates was shaken with 1ml of 1% aluminum chloride solution and observed for light yellow coloration. A yellow precipitate indicates the presence of flavonoids.

Test for Phenols

i. **Ferric chloride test:** The fraction of the extract was treated with 5 % ferric chloride and observed for the formation of deep blue or black colour.

ii. **Liebermann's test:** The extract was heated with sodium nitrite, added H2SO4 solution diluted with water and excess of dilute NaOH was added and observed for the formation of deep red or green or blue colour.

Test for Glycosides

Dilute sulphuric acid (5ml) was added to 0.1g each, of the extracts in a test tube and boiled for 15 minutes in a water bath. It was then cooled and neutralized with 20% potassium hydroxide solution. A mixture, 10ml of equal parts of Fehling's solution A and B was added and boiled for 5 minutes. A more dense red precipitate indicates the presence of glycoside.

Test for Steroids and Terpenoids

A quantity (9ml) of ethanol was added to 1g each of the extracts and refluxed for a few minutes and filtered. Each of the filtrates was concentrated to 2.5ml in a boiling water bath. Distilled water, 5ml was added to each of the concentrated solution, each of the mixtures was allowed to stand for 1 hour and the waxy matter was filtered off.

Each of the filtrates was extracted with 2.5ml of chloroform using a separating funnel. To 0.5ml each of the chloroform extracts in a test tube was carefully added 1ml of concentrated sulphuric acid to form a lower layer. A reddish-brown interface shows the presence of steroids. To another 0.5ml each of the chloroform extract was evaporated to dryness on a water bath and heated with 3ml of concentrated sulfuric acid for 10 minutes in a water bath. A grey colour indicates the presence of terpenoids.

Test for Saponins

A quantity (0.1g) each of the extract (ethanol) was boiled with 5ml of distilled water for 5 minutes. The mixture was filtered while still hot and the filtrates used for the following tests:

i. Emulsion Test: A quantity (1ml) each of the filtrates was added drops of olive oil. The mixture was added to another two drops of olive. The mixture was shaken and observed for the formation of emulsion.

ii. Frothing Test: A quantity (1ml) of the different filtrates was diluted with 4ml of distilled water. The mixture was shaken vigorously and then observed in standing for a stable froth.

Test for Tannins

A quantity (2g) each, of the extracts (n-hexane and water) was boiled with 5ml of 45% ethanol for 5 minutes. Each of the mixtures was cooled and filtered. The different filtrates were subjected to the following tests.

i. Lead Sub-acetate Test: To 1ml of the different filtrates was added 3 drops of lead sub-acetate solution. A cream gelatinous precipitate indicates the presence of tannins.

ii. Ferric Chloride Test: A quantity (1ml) each of the filtrates was diluted with distilled water and added 2 drops of ferric chloride. A transient greenish to black color indicates the presence of tannins.

Test for Proteins

A quantity (5ml) of distilled water was added to 0.1g each, of the extracts. This was left to stand for 3 hours and then filtered. To 2ml portion of the filtrate was added 0.1ml Millon's reagent. It was shaken and kept for observation. A yellow precipitate indicates the presence of proteins.

Burette Test: A quantity (2ml) each of these extracts was put in a test-tube and 5 drops of 1% hydrated copper sulphate was added. A quantity, 2ml of 40% sodium hydroxide was also added and the test-tube shaken vigorously to mix the contents. A purple coloration shows the presence of proteins (presence of two or more peptide bonds).

Test for Carbohydrate

A quantity of 0.1g each of the extracts was shaken vigorously with water and then filtered. To the aqueous filtrate was added a few drops of Molisch reagent, followed by vigorous shaking again. Concentrated sulphuric acid, 1ml was carefully added to form a layer below the aqueous solution. A brown ring at the interface indicates the presence of carbohydrate.

Acute oral toxicity study

Acute oral toxicity was conducted as per the Organization of Economic Cooperation and Development (OECD) guidelines 423 (Acute Toxic Class Method). The acute toxic class method is a stepwise procedure with 3 animals of a single sex per step. Depending on the mortality and /or moribund status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use of a minimal number of animals while allowing for acceptable data based scientific conclusion. The method uses defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemicals which cause acute toxicity. Wistar albino rats of either sex weighing 200-250 g were fasted overnight, but allowed water *ad libitum*. Since *Alpinia galanga* is relatively nontoxic in clinical practice where the rhizome powder 1-3 gm is used[5]. The highest dose of 2000mg/kg/p.o. directly (as per OECD guidelines "Unclassified") was used in the acute toxicity study[6]. The animals were observed closely for behavioral toxicity, if any by using functional observation battery which includes central nervous system signs of excitation, depression, motor activity and autonomic nervous system.

Biochemical studies

The procedures were followed as per standard laboratory technology. Estimation of glucose Aspartate aminotransferase (AST) Alanine aminotransferase (ALT) Alkaline phosphatase (ALP) Urea was estimated using a commercial Glucose estimation kit (Span Diagnostics) by standard methods [7].

Haematological studies

Erythrocyte count, Total Leukocyte Count (WBC) and Hemoglobin was estimated by Hemocytometer method[8].

Statistical analysis

Total 3 rodents are used as per the guidelines provided by OECD 423 and the results were expressed as mean ± standard error of mean (S.E.M.)

RESULTS AND DISCUSSION

Phytochemical analysis

Alpinia galanga belongs to the family Zingiberaceae. The ethanolic extract of Alpinia galanga had an extractive yield of 2.24% with total ash value of 6.17 %, water soluble ash 2.26 % and acid insoluble ash of 3.78% (Table-1). Qualitative phytochemical analysis revealed the presence of various constituents such as alkaloids, carbohydrates, saponins, tannins, protein, glycosides, flavonoids, steroids and terpinoids. Quantitative Phyto chemical analysis of ethanolic extract of *Alpinia galanga* is reported to have maximum total phenol and flavonol content[9]. The results for qualitative Phyto chemical analysis were tabulated in Table no -2.

Table:1. Physical properties extractive values of ethanolic extract of *A. galanga*

S. No	Parameters	Results
1	Loss of drying @ 100 degree Celsius	0.42 %
2	Total ash value	6.17 %
3	Water soluble ash	2.26 %
4	Acid insoluble ash	3.78 %
5	pH at 10% aqueous solution	3.04 %
6	extractive yield - solvent ethanol	2.24%

Table: 2. Preliminary Qualitative Phytochemical study of ethanolic extract of *A.galanga*

Sl No	Constituents	Test	Observation	Inference & Intensity
1	Alkaloids	Mayer's reagent	Milky precipitate	+
		Wagner's reagent	Reddish brown precipitate	+
		Picric Acid (1%)	Yellow precipitate	+
		Dragendorff's test	Red precipitate	+
		Molisch reagent	Dull violet colour	+++
2	Carbohydrates	Fehling A&B	Red precipitate	++
		Benedict's reagent		+++
		Liebermann Burchard	Dark green colour	+++
3	Steroid& Terpenoids	Burette Test	Purple coloration	++
4	Protein	Ninhydrin test	Purple coloration	+++
		Ferric chloride test	Deep blue colour	++
5	Phenols	Liebermann's test	Deep green colour	+
		10% lead acetate	No changes	_
6	Tannins	Braymer's test	No changes	_
		Ammonium Test	Yellow colouration	+
7	Flavanoids	Aluminum Chloride	Yellow precipitate	++
		Glacial acetic acid, $FeSO_4$, Con H_2SO_4	Red precipitate	+++
8	Glycosides	Foam test	No foam	_
9	Saponin	Emulsion test	No emulsion	_

(+) denotes the presence of the respective class compound, (-) denotes the absence of the respective class compound

From this study, the presence of phenolic compounds such as terpenoids, steroids (phytosterols i.e. ß-sitosterol) in ethanolic extract of *Alpinia galanga* may contribute to the antioxidant properties benefited by traditional medicine use. For many years now, it has been

known that plant polyphenols (steroids, terpenoids, flavonoids etc) are antioxidants in vitro[10]. These antioxidants are compounds that reduce the formation of free radicals or react with and neutralize them thus potentially protecting the cell from oxidative damage [11]. The tannins and resins in the extract are employed as astringent both in the gastrointestinal tract and on skin abrasions by traditional medicine. The flavonoids and phenolic compounds in plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti inflammatory, an anti carcinogenic activity which are detected in extract [12].

Acute Toxicity

The acute toxicity study was done as per the guidelines laid by The Organization for Economic Co-operation and Development (OECD). The OECD Test Guidelines are recognized world-wide as the standard reference tool for chemical testing. Since available literature information suggests that mortality is unlikely at the highest starting dose level (2000 mg/kg body weight), the limit test is performed as explained in materials and methods. There was no gross difference in body weight of the control and extract treated group, observed for over the period of 14 days (Table-3).

Table:3. Body weight, Death and Toxicity observations of EE *Alpinia galanga*

Animal identity (markings)	BODY WEIGHT (gm)			Date of death prior to sacrifice	Signs of toxicity (Day 0-14)	Body Weight changes(gm) (Day 0 & 14)
	Initial	**Day-7**	**Day-14**			
HEAD	23.24	23.30	23.30	No death	Nil	00.06
NECK	23.32	23.35	24.37	No death	Nil	00.05
TAIL	24.90	24.95	24..95	No death	Nil	00.05
EE *Alpinia galanga*						
HEAD	24.80	24.80	24.86	No death	Nil	00.06
NECK	25.50	25.55	25.57	No death	Nil	00.07
TAIL	24.00	24.05	24..08	No death	Nil	00.08

Similarly all six animals three in each group are individually observed daily for position, activity, food, water intake and signs of toxicity like hyperactivity, circling, jumping/hopping, excessive grooming, kicking of rear legs, standing on front legs and gait abnormalities. The observation revealed normal activity in both groups (Table -4)

Behavioral hematological and biochemical studies

Gross behavioural studies of ethanolic extract of A.*galanga* – 2000mg/kg and control group observed for first four hours, twenty four hours, day 7 and day 14 for central nervous system excitation, depression, autonomic nervous system and motor activity. The extract revealed normal behavioral activity. At the end of 14 days the control and ethanolic extract of A.*galanga* treated group was sacrificed and investigated for haematological, biochemical and necropsy analysis. The results revealed no significant difference in haematological, biochemical parameters (Table-5 & 6) and in necropsy observation of sacrificed animals did not show any gross abnormalities in tissues and major organ.

Table:4. Individual cage -side observations

Animal identity (markings)	Position	Activity	Food and Water intake	Signs of toxicity	Onset of toxicity
Control					
HEAD	Normal	Normal	Adequate	Nil	Nil
NECK	Normal	Normal	Adequate	Nil	Nil
TAIL	Normal	Normal	Adequate	Nil	Nil
EE *Alpinia galangal*					
HEAD	Normal	Normal	Adequate	Nil	Nil
NECK	Normal	Normal	Adequate	Nil	Nil
TAIL	Normal	Normal	Adequate	Nil	Nil

Table:5. Effect of EE *Alpinia galanga* rhizome on hematological parameters after 14 days of acute toxicity testing in Swiss albino mice

Groups (n=3)	Hb % (gm)	RBC (milli/cu.mm)	WBC (cells/cu.mm)	Differential leukocyte count (%)		
				Lymphocytes	**Monocytes**	**Granulocytes**
Control	15.03 ± 2.49	10.85 ± 1.63	7.05 ± 0.56	71	19	8.9
EE Alpinia galanga	15.61 ± 3.46	11.68 ± 1.48	7.96 ± 1.59	75	15	9

Table:6. Effect of EE *Alpinia galanga* rhizome on biochemical markers after 14 days of acute toxicity testing in Swiss albino mice

Groups	**AST (IU/L)**	**ALT (IU/L)**	**Urea (mg/dl)**	**Creatinine** (mg/dl)	**Glucose (mg/dl)**
Control (D. W)	45.50 ± 3.19	14.40 ± 0.73	56.07 ± 2.06	0.48 ± 0.40	286.04 ± 3.96
EE *Alpinia galangal*	47.45 ± 2.44	13.78 ± 1.65	54.04 ± 1.78	0.48 ± 0.35	279.71 ± 18.39

CONCLUSION

Selection of scientific and systematic approach to the biological evaluation of plant products based on their use in the traditional system of medicine forms the basis for an ideal approach in the development of new drugs from the plant [13]. Phytochemical screening of *Alpinia galanga* has revealed the presence of many complex substances including phenols and flavanoids which are responsible for many biologic activity and acute toxicity test in the present study has revealed that the ethanolic extract of *Alpinia galanga* substance is classified in the hazard category 2000mg/kg < LD50 < 5000mg/kg according to globally harmonized classification system.

ACKNOWLEDGEMENTS

The Author profoundly thanks the Vice principal Dr M. Chandrasekhar Meenakshi Medical College and Research Institute, and Mr. Purushothaman, for providing assistance in utilizing central research lab facilities.

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