RESEARCH PAPER

Pharmacognostic, Phytochemical Investigation with Evaluation of Acute Toxicity of Polyherbal Extract

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ABSTRACT
Polyherbal formulation have gained significant importance in both traditional and modern medicine due to their reduced side effects and enhanced synergistic therapeutic potential. The present study aims to establish the pharmacognostic and phytochemical profile of a novel polyherbal formulation and to evaluate its safety through an acute oral toxicity study in experimental animals. Pharmacognostic analysis, including macroscopic and microscopic characterization was performed to establish the identity, purity and quality of the constituent plant materials. Physicochemical parameters such as ash values, extractive values, and moisture content were also determined following standard procedures. Preliminary phytochemical screening of various solvent extracts along with acute toxicity study was also carried out as per standard guidelines.

Keywords: Polyherbal formulation, Pharmacognostic, Phytochemical screening, Acute toxicity, Physicochemical characteristics
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INTRODUCTION

India is rich source of medicinal plants both cultivated and wild employed for treatment of various ailments. The different parts of plant use for this purpose are flower, fruit, root, stem, foliage and seed which can be used as such or in the form of extracts containing chemical compounds can be isolated from them to produce drugs for human and veterinary medicine[1,2].

In spite of many advancements in the field of medicine plants still remain the main source of drug[3].The World Health Organization (WHO) has included list of over 20,000 medicinal plants, used in different parts of world for the treatment of wide range of diseases. This practice is in use since ancient times (Ayurveda, Siddha, and Unani-Tibb) and still continues to serve together with modern medicine for large portions of population in developing countries.

These plants contain bioactive constituents like alkaloids, flavonoids, glycosides, tannins, and phenolics which impart diverse pharmacological properties and increased interest in natural and plant- based drugs worldwide has led to extensive research on medicinal plants with better therapeutic action, low cost, easy availability and reduced side effects[4].

Plants description

Acacia nilotica (L.) belonging to family Fabaceae is commonly found in tropical and sub-tropical regions has enormous medicinal values with every part of plant possessing bioactive secondary metabolites that can be used for the development of new drugs and also for the treatment of various human diseases such as Diabetes, Alzheimer's, Diarrhoea, Hypertension, Asthma, Ulcer, Leprosy, Small pox, Bleeding piles and Leucoderma. It is also known as Indian gum Arabic, black babool, acacia arabica, babhul or babool [5,6,7]

Andrographis echioides (L.) belonging to family Acanthaceae also known as false willow is dicotyledon plant native to Asia and endemic in India. The leaves of plant were used in Siddha and Ayurveda for the treatment of ulcer and inflammation.The plant also exhibit diuretic, analgesic, hepatoprotective and anti-oxidant anti-allergenic, anti-microbial properties[8,9].

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Canna indica (L.) belonging to family Cannaceae commonly known as Indian shot or canna lily is ornamental plant native to tropical regions of America later in countries like India and Sri Lanka. The different parts of plant have been used for treatment of wounds, malaria, diarrhea, dermatosis[10,11].

MATERIAL AND METHODS

The pharmacognostic investigations were carried out on the polyherbal formulation of

Acacia nilotica	**Family:** *Fabaceae*
Adrographis echioides	**Family:** *Acanthaceae*
Canna indica	**Family:** *Cannaceae*

Plant collection and Authentication:

The leaves of Acacia nilotica, Andrographis echioides and Canna indica were collected from the local area of Hyderabad then washed with tap water to remove dust, soil and other impurities. The collected leaves were then shade dried, powdered and sieved through mesh No.40 and stored in closed container. The specimen was identified and authenticated by Dr. L. Rasingam, Scientist -In- charge, Botanical survey of India, Deccan regional centre, Hyderabad, Telangana state, India.

Pharmacognostic evaluation

1. Determination of leaf constants[12]

a. Stomatal number

Definition: Stomatal number is the average number of stomata per square mm of epidermis of leaf

Procedure: The middle part of leaf was clarified by boiling it in chloral hydrate solution and the upper and lower epidermis were peeled separately by means of forceps.

Then peeled epidermis was placed on glass slide and mounted with glycerine. The pictomicrograph was taken with the help of digital microscopic eyepiece attached with microscope and the number of stomata were then counted.

b. Stomatal index

The stomata index was calculated using the formula

$$I = \frac{S}{S + E} \times 100$$

Here I= Stomata index

S= Number of stomata

E= Number of epidermal cells

c. Palisade ratio

Definition: Palisade ratio is defined as the average number of palisade cells beneath single upper epidermal cell.

Procedure: The leaf was cleared by boiling in chloral hydrate solution and mounted under microscope, then count the palisade cells under the four epidermal cells. Repeat it to obtain five different readings from different parts of the leaf in order to obtain the accurate average which is the palisade ratio.

d. Vein islet number

Definition: It is the average number of veinlets enclosing in 1sq. mm of leaf surface area.

Procedure: The lamina of leaf between midrib and margin was cut into pieces and then boiled in chloral hydrate solution till the leaf gets discoloured and then the slide was prepared and observed under microscope for vein islets in 1 mm sq. area. For accurate determination five readings were taken[13].

e. Veinlet termination number

Definition: Veinlet termination number is the average number of terminated veinlets in 1 sq. mm of leaf surface area.

Procedure: Same like the determination of vein islet number, count the number of vein termination in 1 mm sq. area[12].

2. Physicochemical characteristics of selected polyherbal plants

a. Determination of ash values[14]

The identified crude drug can be of substandard quality either due to faulty collection or incorrect storage and in order to prove its acceptability as a drug the following tests can be applied. After incineration the residue which remains is the ash content of the drug which can be inorganic salts occurring naturally, or adhering to it or added as adulterant.

i. Determination of total ash

Total ash consists of both physiological (derived from plant tissues) as well as non- physiological ash (obtained from residue of extraneous matter, sand, soil, etc)

Procedure: Nickel crucible was tarred and placed in muffle furnace for 15 minutes at 450°C then cooled in desiccator for 1 hr and weighed as W_1

Then 3.0gm of powdered drug was placed in the crucible and heated until all the moisture has been removed and plant material gets completely charred (W_2). The heat was increased slowly to 650°C until the carbon gets vaporized and the sample turns grey (white ash). The material was then cooled in desiccator and weighed to calculate total ash[12].

ii. Determination of acid insoluble ash

Procedure: To the total ash obtained 25 ml of hydrochloric acid was added and boiled for 5 minutes then the insoluble matter was collected on ashless filter paper after washing with hot water, incinerated until free from carbon. Later it was cooled in a desiccator and weighed to calculate acid-insoluble ash[15].

iii. Determination of water-soluble ash

Procedure: The obtained ash was boiled with 25ml of distilled water for 5 minutes. The insoluble matter was collected on ashless filter paper or in crucible then washed with hot water, ignited and weighed to calculate the amount of water-soluble ash[16].

b. Determination of loss on drying About 5gm of drug was accurately weighed in petri dishes and was kept in hot air oven for 4 hrs at 110°C until constant weight is achieved. then it was transferred to desiccators for cooling and weighed[17].

$$Loss\ on\ drying = loss\ in\ weight\ of\ sample/weight\ of\ sample \times 100$$

c. Determination of extractive values (alcohol soluble extractive, water soluble extractive, petroleum ether soluble extractive, chloroform soluble extractive)

A 5.0gm of material was weighed accurately and was transferred to stoppered conical flask then different solvents (alcohol, water, petroleum ether, chloroform) were added separately and the stopper of conical flask was replaced firmly. The flask with its contents were shaken mechanically for 6hrs followed by maceration for another 18hrs and then filtered through Whatmann No. 1 filter paper. The filtrate obtained was evaporated to dryness and the residue was dried to a constant weight at 105° C[12,18].

3. Extraction procedure

Preparation of plant extract: The leaves of Acacia nilotica, Andrographis echioides, and Canna indica were collected and shade dried. Later it was powdered in a mixer to required particle size and sieved through sieve no 40.

About 500 gm of powder material was extracted in Soxhlet apparatus using successive solvents of increasing polarity such as petroleum ether, chloroform, ethanol, and water for 48 hours. The filtrates were collected and evaporated to dryness under reduced pressure using rotary flash evaporator. The extracts obtained from different solvents were weighed and its percentage was calculated in terms of air-dried weight of plant material. Then the dried extracts were preserved at 4° C in small sterilized containers.

i. Preparation of petroleum ether extract

The powder was packed in Soxhlet apparatus and extracted with petroleum ether (60-80°C) for 48 hours. The extract was transferred to china dish and evaporated to thick paste on water bath maintained at 50°C to get the petroleum ether extract. The marc was then air dried thoroughly to remove the solvent before it was taken for further extraction with next solvent.

ii. Preparation of chloroform extract

The air-dried powder from the above process was extracted successively with chloroform to get chloroform extract. The marc was then collected, dried and used for preparing further extraction with ethanol.

iii. Preparation of ethanol extract

The dried chloroform marc was successively extracted with ethanol to get ethanolic extract. The marc obtained from this step was collected, dried and used for further investigation.

iv. Preparation of aqueous extract

2000 gm of powder was taken in 5000ml beaker and macerated with 3000ml of distilled water and 100ml of chloroform (as preservative) for 7 days. The marc from this step was concentrated at 50° C on water bath to get semi solid mass.

The percentage yield from all the extracts was calculated and tabulated.

v. Preliminary phytochemical analysis of extracts[19]

S.No	TEST	OBSERVATION	INFERENCE
1.	**Test for carbohydrates:** a) **Benedicts test:** 0.5ml extract+5ml Benedict's reagent b) **Fehlings test:** 2ml extract+1ml equal part of Fehlings A and B and then boil **c) Molischs test:** 2ml extract + 2 drops of 20% α-napthol + 2ml of conc. sulphuric acid	Red, yellow, or green precipitate is observed Formation of red or brick red precipitate Formation of violet at the junction of solution	Indicates presence of reducing sugar
2.	**Test for Proteins** **a) Biurets test:**1ml extract+ 1ml 10% NaOH + drop of $CuSO_4$ **b) Ninhydrin test:** few ml extract + 0.5ml ninhydrin reagent and boil for 2mins	Formation of purplish violet colour Appearance of purple colour	Indicates the presence of protein
3.	**Test for alkaloids** **a) Wagners test:** 1ml extract+ 1.5% HCl+ 5 drops Wagners reagent **b) Meyers test:** 1ml of extract+ 5 drops of Meyers reagent	Formation of brown or reddish-brown precipitate Formation of yellow cream precipitate	Indicates the presence of alkaloids
4.	**Test for flavonoids** **a) Alkaline reagent test:** few drops extract+ few drops NaOH solution **b) Shinodas test:** 0.5ml extract + 5 to 10 drops conc. HCl+ piece of	Formation of intense yellow colour, which turns colourless with addition of dil. HCl Formation of magenta	Indicates the presence of flavonoids

	magnesium then boil	colour	
5.	**Test for phenols:** **a) Ferric chloride test:** 1ml extract + few drops 10% $FeCl_3$ solution **b) Lead acetate test:** 1ml extract+ 3ml distilled water+ 5 drops aqueous lead acetate **c) Libermanns test:** extract+ 0.5ml 20% H_2SO_4 + few drops aqueous sodium nitrate solution	Formation of blue, green or violet colour Formation of yellow precipitate Red colour is observed	Indicates the presence of phenols
6.	**Test for saponins:** **Foam test:** extract+ few drops $NaHCO_3$ solution and shake vigorously then leave for 3 mins	Honey comb like worth is observed	Indicates the presence of saponins
7.	**Test for tannins** **a) Ferric chloride test:** 1ml extract+ few drops $FeCl_3$ **b) Lead acetate test:** 5ml extract + 5 drops 1% lead acetate	Formation of bluish black colour which disappears with addition of few ml dil. H_2SO_4 and changes to yellowish brown Yellow or red precipitate is formed	Indicates the presence of tannins
8.	**Test for glycosides** a) **Legals test:** extract + 1ml sodium nitroprusside solution + NaOH solution **b) Keller killani test:** extract+ 1ml water + NaOH solution	Appearance of pink to red colour Formation of yellow colour precipitate	Indicates the presence of glycosides
9.	**Test for steroids** **Salkowkis test:** 2ml extract+ 1ml conc. H_2SO_4	Formation of red colour in the chloroform layer	Indicates the presence of steroids

Acute toxicity study

Acute toxicity study involves the administration of single dose of drug in large quantity to determine the immediate toxic effect or LD_{50} of drug or chemical and natural products[20].

Test animals: Young and healthy Swiss albino mice of the weight about 24-25 g were selected for the study and were acclimatized to the laboratory environment for a week at 22±5° C with humidity control and 12hrs dark and light cycle. All the animals were fed with standard pellet diet, water ad libitum, test substances through gastric gavage[21,22].

Procedure: The mice were fasted 24 h prior to the commencement of the study and were divided into 4 groups consisting of 3 animals each receiving dose levels 5, 50, 300, and 2000 mg/kg respectively. After dosing the animals were observed for any mortality, behavioural, autonomic and toxic profile changes for one to four hours and up to 14 days, at least once daily for the immediate and delayed acute toxicity as the parameters specified below during the monitoring period.

1. **Motor activity**
2. **Grooming**
3. **Touch response**
4. **Pain response**
5. **Tremors**
6. **Convulsions**
7. **Gripping strength**
8. **Pinna reflex**
9. **Corneal reflex**
10. **Writhing**
11. **Pupils**
12. **Urination**
13. **Salivation**
14. **Skin colour**
15. **Lacrimation**

RESULTS

Determination of leaf constants:

S.No	PARAMETER STUDIES	ACACIA NILOTICA	ANDROGRAPHIS ECHIOIDES	CANNA INDICA
1.	Stomata	2	2	3

	number			
2.	Stomatal index	25	25	27
3.	Palisade ratio	1:4	1:4	1:5
4.	Vein islet number	4-7	4-6	4-9
5.	Veinlet termination number	3-6	3-5	3-8

Physicochemical characteristics:

S.No	PHYSICOCHEMICAL CHARACTERISTICS	RESULT (%w/w)		
		Acacia nilotica	Andrographis echioides	Canna indica
1.	Total ash	13.79	13.02	14.63
2.	Acid- insoluble ash	2.41	2.29	2.94
3.	Water-soluble ash	2.19	1.98	2.45
4.	Loss on drying	9.90	9.31	10
5.	Alcohol (ethanol) soluble extractive	11.59	12.55	12.13
6.	Water soluble extractive	70.01	67.73	77.80
7.	Petroleum ether soluble extractive	4.75	5.58	5.10
8.	Chloroform soluble extractive	4.70	5.56	5.05

Percentage yield of successive extract of Acacia nilotica, Andrographis echioides, Canna indica

S.No	EXTRACTS		NATURE OF EXTRACTS	COLOUR	WEIGHT (gm)	PERCENTAGE YEILD
1.	AN	PETROLEUM ETHER	All extracts are in the nature of semi solid	Dark green	100.8	10.08
	AE			Dark green	95.2	9.52
	CI			Dark green	110.5	11.05
2.	AN	CHLOROFOM		Dark green	110.79	11.07
	AE			Dark green	112.10	11.21
	CI			Dark green	105.21	10.52
3.	AN	ETHANOL		Dark Brown	270.68	27.06
	AE			Dark Brown	280.19	28.01
	CI			Dark Brown	296	29.6
4.	AN	AQUEOUS		Dark Brown	210.90	21.09
	AE			Dark Brown	205	20.5
	CI			Dark Brown	218.30	21.83

Preliminary phytochemical analysis of successive extracts of Acacia nilotica, Andrographis echioides, Canna indica

S.No	Phytochemical constituent	Petroleum ether extract			Chloroform extract			Ethanol extract			Aqueous extract		
		AN	AE	CI	AN	AE	CI	AN	AE	CI	AN	AE	CI
1.	Alkaloids	-	-	-	-	-	-	+	+	+	-	+	+
2.	Flavonoids	-	-	-	-	-	-	++	++	++	++	++	++
3.	Saponins	-	-	-	-	-	-	+	+	+	++	++	++
4.	Tannins	-	-	-	-	-	-	+	+	+	+	+	+
5.	Phenols	+	+	+	+	+	+	+	+	+	++	+	+
6.	Steroids	-	-	-	-	-	-	+	+	+	+	+	+

7.	Glycosides	-	-	-	-	-	-	+	+	+	+	+	+
8.	Carbohydrates	-	-	-	-	-	-	-	-	-	++	++	++
9.	Amino acids	-	-	-	-	-	-	+	+	+	+	+	+

AN= Acacia nilotica

AE=Andrographis echioides

CI= Canna indica

+=Indicates presence of phytochemical

_ = Indicates absence of phytochemical

++ = Indicates high concentration of phytochemicals

Acute oral toxicity

S.No	Response	Different concentrations of polyherbal extracts															
		5mg/kg				50mg/kg				300mg/kg				2000mg/kg			
		PE	CE	EE	AE	PE	CE	EE	AE	PE	CE	EE	AE	PE	CE	EE	AE
1.	Motor activity	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
2.	Grooming	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
3.	Touch response	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
4.	Pain response	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
5.	Tremors	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
6.	Convulsion	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
7.	Gripping strength	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
8.	Pinna reflex	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
9.	Corneal reflex	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
10.	Writhing	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
11.	Pupils	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
12.	Urination	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
13.	Salivation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
14.	Skin colour	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
15.	Lacrimation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

A= Absent

P= Present

N= Normal

CONCLUSION

Pharmacognostic evaluation serves as a tool for proper collection, identification and investigation of the plants which further confirms the quality and purity of plant. The findings of the present study suggest that the polyherbal formulation of Acacia nilotica, Andrographis echioides and Canna indica is pharmacognostically standardized and is rich source of secondary metabolites such as proteins, amino acids, steroids, alkaloids, phenolics, flavonoids, and tannins which can be utilized for several medicinal and therapeutic purposes. Furthermore, no mortality or significant clinical signs of toxicity (such as convulsions, tremors or salivation) were observed at the single oral limit dose of 2000 mg/kg. Besides the body weight and food/water intake remained normal compared to the control group the result of acute toxicity clearly indicates that the polyherbal plant extract is safe and non- toxic up to a dose of 2000mg/kg suggesting lethal dose (LD_{50}) to be greater than 2000mg/kg. Hence the study provides scientific basis for the traditional use of formulation and supports its further pharmacological and clinical evaluation.

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