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Research Article

PHYTOCHEMICAL PROFILE AND ACUTE TOXICITY STUDIES OF ETHANOLIC EXTRACT OF *SYZYGIUM ALTERNIFOLIUM* (WIGHT) WALP.

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ABSTRACT

Syzygium (Myrtaceae) is among the largest genus of Dicotyledonae which is pan global with about 1,200 species reported from around the world. Owing to the unverified claims made on the medicinal properties and therapeutic efficacy of *Syzygium alternifolium,* this study pursued with fruit extracts receives a special impetus. Ethanol extract made by continuous hot percolation method was tested for its phytochemical constituents and were evaluated for its toxic effects. Though responded negatively in the tests for carbohydrates, gums, mucilage, tannins ad phenolics, the ethanolic fruit extract is worth referring as a therapeutic as there is rich haul of other potentially curative secondary metabolites. Analysis of Ethanolic Extract of *Syzygium alternifolium* (EESA) by GC-MS showed the presence of various components such as Glycerin, Pentanoic acid, 4-oxo-, 4H-Pyran-4-one, 2,3- dihydro-3,5- dihydroxy-6- methyl-,2- Furancarboxaldehyde,5-(hydroxymethyl)-,1-octanol,2-butyl-,2,5-monomethylene-1-rhamnitol 1,6 anhydro-a-D-galactofuranose, 1,6 anhydro-a-D-glucofuranose, Hexanedioic acid, bis (2-ethylhexyl) ester, 1,2-Benzene dicarboxylic acid, diisooctyl ester and Squalene. In this present study performed on acute oral toxicity testing in rats, a single dose of 2 g/kg of the extract could be placed under acute toxicity class 5- > 2000 mg- 5000 mg/kg (OECD Guidelines, 2000). These findings provide information on the doses to be given in the subsequent pharmacological studies as the fruit extracts are presumed to treat a number of common and critical aliments.

Kew words: *Syzygium alternifolium,* Myrtaceae, Phytochemical compounds, GC-MS analysis, Acute toxicity

INTRODUCTION

Peninsular India endowed with the Western and Eastern Ghats is a safe haven for a number of endemic species with medicinal value. Several species in *Syzygium* genera are mostly available in Western Ghats of India. But *Syzygium alternifolium* present especially in mountain range of the Middle Eastern Ghats of India. *Syzygium alternifolium* (Wight) Walp. is an Indian endemic medicinal species of the popular genus *Szygyium S. alternifolium* is sparsely reported and about less 20 individual trees have been spotted in Southern Eastern Ghats region of Alagarkoil hills in Madurai. Choosing to survive in mid elevations, the plant familiar in local area, is considered a phyto remedy by local people, even though it's healing properties stands unverified.

S. alternifolium is a fruit tree of timber, medicinal and economic importance. Wood from the tree is used for making furniture and agricultural implements. The plant twigs are used to cure skin diseases as it has excellent anti-fungal properties and antidiabetics[1]. Leaves find their use in the treatment of liver cirrhosis, hepatitis, infective hepatitis, liver enlargement, jaundice and other ailments of liver and gall bladder[2]. When fried in cow ghee tender foliage is used as a curry leaves are claimed to control dry cough. A mixture of leaves and mineral oil is used to maintain dark hair and also to promote hair growth by external application. Tender shoots, fruits and leaf juice are used to treat dysentery, seeds for diabetes and stem bark for gastric ulcer[3]. Flowers yield honey and possess antibiotic properties[4,5]. The ripe fruits are used in making squashes and jellies. Fruit juice is used to cure stomach-ache and ulcers while the external application of fruit pulp reduces rheumatic pains[6]. That the species seen devoured by monkeys for its fruits and the latter sold locally as herbal medicine prompted the need for this study. As part of the study exploring the therapeutic capabilities, this present investigation is made to qualitatively test the phytochemical constitution and also check acute toxicity tests to evaluate the pharmacological analysis for future.

MATERIALS AND METHODS

Fresh fruits of *Syzygium alternifolium* are collected from wild in the fortnightly field trips at the *Syzygium* site at an elevation about 700 MSL on the northern slope of Alagar Hills along the walking trail to Natham. After discarding the insect infested ones, the healthy fruits of the species were shade dried for over two months or until the stage there was no more weight loss due to evapo-transpiration. At this stage the fruits were pulverized in pestle and mortar first and then change into a dry powder through mixer grinder. Dry powder of sample about 250 gram was set apart for solvents extraction using Soxhlet apparatus. The consistency of the extract is semi solid. This method is repeated until getting desired extract. This extract is subjected to further analysis for Qualitative Phytochemical test and acute toxicity also. The voucher specimen is preserved as herbarium (ACH-349) in department of botany, The American College, Madurai. The identification was done with the help of Dr. D. Stephen Assistant Professor, Department of Botany, The American College, Madurai, Tamil Nadu, India.

Qualitative Phytochemical Analysis

Among the different fraction gathered in the study, the ethanolic extract of *Syzygium alternifolium* was subjected to various tests for identification of constituents. Preliminary phytochemical tests were performed as indicated below[7].

Detection of Carbohydrates

Small quantities of Ethanolic extract of *Syzygium alternifolium* was dissolved in distilled water separately and filtered. The filtrates were taken for the various tests to detect the presence of carbohydrates.[8]

Molisch's Test

The filtrates were treated with 2-3 drops of 1% alcoholic α-napthol and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. The expected brown ring was not formed in Ethanolic extract of *Syzygium alternifolium*.

Fehling's Test

Small portion of the filtrates were treated with equal volume of Fehling's solution A and B and then heated. A brick red precipitate not formed in the presence of sugars

Benedict's Test

Small portion of the filtrates were treated with equal volume of Benedict's reagent. A yellow precipitate was not formed indicating negative result with Ethanolic extract of *Syzygium alternifolium*

Barfoed's Test

Small portion of the Ethanolic extract of *Syzygium alternifolium* were treated with Barfoed's reagent. Red precipitate did not emerge in the ethanolic extract of the plant

Test for Starch

A small amount of the Ethanolic extract of *Syzygium alternifolium* was treated with dilute iodine solution. That bluish black color was not observed in the Ethanolic extract of *Syzygium alternifolium* indicates the absence of starch.

Tests for Gums and Mucilage's

The Ethanolic extract of *Syzygium alternifolium* were treated with absolute alcohol, stirred and filtered. The filtrate was dried and examined for its swelling properties. Since ethanolic extract of *Syzygium alternifolium* restrained evincing changes in inhibition properties gums and mucilage were considered absent in the tested sample.

Test for Proteins and Amino Acids

Small quantities of Ethanolic extract of *Syzygium alternifolium* was dissolved in few ml of distilled water and subjected to Ninhydrin test, Xanthoprotein test for presence of proteins and amino acids in extract.

Ninhydrin Test

Ethanolic extract of *Syzygium alternifolium* were treated with ninhydrin reagent (0.1% solution) and boiled. Purple color was observed indicating the presence of protein in Ethanolic extract of *Syzygium alternifolium*

Biuret Test

To a portion of the above prepared extracts, equal volumes of 5% w/v sodium hydroxide and 4 drops of 1% w/v copper sulphate solution were added. Violet color was formed, indicating the presence of protein in Ethanolic extract of *Syzygium alternifolium.*

Millon's Test of Cole's Mercuric Nitrite Test

To the above-prepared extracts, Millon's reagent was added. White precipitate was formed, showing the presence of protein in the extracts of Ethanolic extract of *Syzygium alternifolium*.

Xanthoprotein Test

To 3 ml of the above-prepared extracts, 1 ml of the concentrated nitric acid was added, boiled for one minute, cooled and concentrated ammonia was added till alkaline. An orange color was formed, showing the presence of protein in Ethanolic extract of *Syzygium alternifolium*.

Test for Fixed Oils and Fats

Spot Test

A small quantity of Ethanolic extract of *Syzygium alternifolium* was pressed between two filter papers. Oil stains were observed with the extracts indicating the presence of fixed oils and fats.

Saponification Test

Few drops of 0.5 N alcoholic potassium hydroxide was added Ethanolic extract of *Syzygium alternifolium* along with of few drops of phenolphthalein. The mixture was heated on a water bath for one hour. Soap was formed with the extracts indicating the presence of fixed oils and fats.

Test for Alkaloids

Small amount of the solvent free Ethanolic extract of *Syzygium alternifolium* were separately stirred with a few ml of dilute HCl and filtered. The filtrates were tested with various alcoholic reagents.

Mayer's Test

To the small quantities of the extracts, Mayer's reagent was added. Presence of cream-colored precipitate indicates the presence of alkaloids in Ethanolic extract of *Syzygium alternifolium*.

Dragendroff's Test

To small quantity of extracts, Dragendroff's reagent was added. Presence of orange brown precipitate indicates the presence of alkaloids.

Wagner's Test

To small quantity of the extracts, Wagner's reagent was added. Presence of reddish-brown precipitate, indicate the presence of alkaloids.

Hager's Test

To small quantity of the extracts, Hager's reagent was added. Presence of yellow precipitate indicate the presence of alkaloids.

Tests for Glycosides

A small amount of the Ethanolic extract of *Syzygium alternifolium* were dissolved separately in 5 ml of distilled water and filtered. Another portion of the extracts were hydrolyzed with hydrochloric acid for one hour on a water bath and hydrolyzed was subjected to Legal's, Baljet's, Borntrager's, Keller-Killani's tests and for the presence of cyanogenetic glycosides.[9]

Legal's Test
To the hydrolyzed, 1 ml of pyridine and a few drops of sodium nitroprusside solution was added and then made alkaline with sodium hydroxide solution. Pink color was observed in three extracts.

Baljet's Test
To a section of plant extract, sodium picrate solution was added. Yellowish orange color was observed in Ethanolic extract of *Syzygium alternifolium*.

Borntrager's Test
Hydrolyzed was treated with chloroform and the chloroform layer was separated. To this, equal quantity of dilute ammonia solution was added. Pink color was observed in the ammonical layer of chloroform in Ethanolic extract of *Syzygium alternifolium* showed the presence of glycosides.

Test for DeoxySugar (Keller-Kiliani Test)
To the extracts 10 ml of 70% alcohol were added, boiled on a water bath, filtered. The filtrates were diluted with 1 ml of distilled water; 1 ml of strong lead acetate solution was added and filtered. The filtrates were extracted with an equal volume of chloroform. The chloroform layer was pipette out and evaporated to dryness. The residue obtained was dissolved in 3 ml of 3.5% of ferric chloride in glacial acetic acid, left for one minute and then transferred to a test tube. To the side of the test tube, 1.5 ml of sulphuric acid was added carefully, which formed a separate layer at the bottom and kept for few minutes.

Blue color at the interface and pale green color in the upper layer was observed in Ethanolic extract of *Syzygium alternifolium* showed the presence of glycosides.

Test for Phytosterols

Small quantities of the extract were dissolved in the 5 ml of chloroform separately. Then these chloroform solutions were subjected to Liebermann's test, Liebermann-Burchard's test, Salkowski's test.[10]

Liebermann-Burchard's Test
The residue was dissolved in chloroform. To this Liebermann-Burchard's reagent was added. Green color was produced. Green color was produced in Ethanolic extract of *Syzygium alternifolium* indicating the presence of phytosterols.

Salkowski's test
A few drops of concentrated sulphuric acid were added to chloroform solution. The lower layer of the solution turned brownish red color with the Ethanolic extract of *Syzygium alternifolium* indicating the presence of phytosterols.

Test for Flavonoids

The Ethanolic extract of *Syzygium alternifolium* were separately dissolved in ethanol and then subjected to the following tests.[11]

Ferric chloride Tests
To a small quantity of the extract, few drops of neutral ferric chloride were added. Blackish red color was observed in Ethanolic extract of *Syzygium alternifolium* indicating the presence of Flavonoids.

Shinoda's test
A small quantity of the extract was dissolved in alcohol and to this magnesium metal followed by concentrated hydrochloric acid, was added drop wise and heated. A magenta color was produced in Ethanolic extract of *Syzygium alternifolium* indicating the presence of Flavonoids.

Flavones

1. With sodium hydroxide solution, the extract gave yellow color.
2. Extract gave orange color with concentrated sulphuric acid.

Reaction with alkali and acid

When alcoholic solution was treated with alkali and acid, yellowish color indicating the presence of Flavonoids

Test for Tannins
The extracts were dissolved in water and filtered. The filtrates were treated with various reagents.[12]

Ferric chloride test
Few ml of the filtrates was treated with 5% ferric chloride solution. A bluish black color was not observed indicating the absence of tannins in Ethanolic extract of *Syzygium alternifolium*

Reaction with lead acetate

Few ml of the filtrates was treated with lead acetate solution. White precipitates were not produced in Ethanolic extract of *Syzygium alternifolium* indicating the absence of tannins.

Gelatin Test
The extracts were dissolved separately in minimum amount of water and filtered. To the filtrate, add 1 ml of 1% solution of gelatin. Extracts did not produce any white precipitate.

Test for Saponins

The extracts were diluted with 20 ml of distilled water and agitated in a graduated cylinder for 15 minutes. One-centimeter layer of foam was formed in extracts indicating the presence of saponins.[13]

Phytochemical analysis through Gas Chromatography – Mass Spectroscopy (GC-MS)

The Gas Chromatography (GC) analysis was carried out by using GCMS-Clarus 500 Perkin Elmer instrument. Gas Chromatography (GC) is equipped mass selective detector coupled to front injector. The column chromatography was filled with Diphenyl and Dimethyl poly siloxane, column thickness (30 X 0.25 mm X 0.25 µm). The injector temperature was set at 250^0C. Helium (He) is used as the carrier gas at constant flow rate 1.21 ml/min. The percentage of composition of extract was calculated by GC peak areas. The compounds were identified based on comparison of their retention time and mass spectra.

Spectral interpretation of GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The spectrum of the unknown component was compared with the spectrum of the known component stored in the NIST library version (2005).

Acute toxicity study

Pharmacological Evaluation was pursued after obtaining the approval of experimental work from IAEC/CPCSEA. The protocol of the animal experiments involved in this research work

has been approved by IAEC/CPCSEA constituted for this purpose. All the experiments are conducted in accordance with the ethical guidelines of the CPCSEA (661/02/C/IAEC/KMCP). The experimental protocol was approved by the Institutional Animal Ethical Committee of K.M. College of Pharmacy, Madurai District, Tamil Nadu.

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours. Acute toxic class method (OECD guidelines, 2000)[14] was followed to arrive at the maximum safety dose of the drug extracts. Three wistar strain female albino rats (8-12 weeks old, 180-200 g body weight) were used in each group. Single dose (2 g/Kg) of the extracts were orally administered to overnight fasted (food but not water withheld) animals while control animals received the vehicle (0.3% w/v CMC). Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 h, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Body weights of the animals were recorded. At the end of 14th days, all animals were subjected to gross necropsy.

RESULTS

On standardization of procedures used for extraction with different solvents based on polarity, yield in each case is assessed and the result is presented (Table 1). With a standard time taken for running the extraction for two consecutive days with an intervening night continuously, the residues were collected and tested for the color and texture. In order to endure that maximum dissolution happens in ethanol, the alcoholic extraction was allowed an extended time of three days.

Table 1: Percentage Yield of Various Extracts of *Syzygium alternifolium*

Solvent used for Extraction	Time required for complete extraction (Hours)	Color of extract	Percentage of yield (w/w)
Petroleum ether	40	Dark green	7.20 %
Benzene	40	Dark greenish brown	9.80 %
Ethyl acetate	40	Dark green	8.50 %
Ethanol	72	Dark brown	12.30 %

Table 2: Qualitative Chemical Analysis of Ethanolic Extract of *Syzygium alternifolium*

S. No.	Test for Plant Constituents	EESA
	a. Molisch's test	
	b. Fehling's test	_
	c. Benedict's test	_
	d. Barfoed's test	_
	e. Test for Starch	_
2	**Test for Gums and Mucilage**	
	Alcoholic precipitation and Molisch's test	
3	**Test for Proteins and amino acids**	
	a. Ninhydrin Test	+
	b. Biuret Test	+
	c. Millon's test or Cole's Mercuric Nitrate test	+
	d. Xanthoprotein test	+
4	**Test for fixed oils and fats**	
	a. Spot test	+
	b. Saponification Test	+
5	**Test for alkaloids**	
	a. Mayer's Test	+
	b. Dragendorff's Test	+
	c. Wagner's Test	+
	d. Hager's Test	+
6	**Test for Glycosides**	
	a. Legal's Test	+
	b. Baljet's Test	+
	c. Borntrager's Test	+
	d. Keller-Kiliani's Test	+
7	**Test of Phytosterols**	
	a. Liebermann-Burchard's Test	+
	b. Salkowski's Test	+
8	**Test for Flavanoids**	
	a. Ferric chloride Test	+
	b. Shinoda's Test	+
	c. Fluorescence Test	+
	d. Reaction with alkali and acid	+
9	**Test for Tannins and Phenolic Compounds**	
	a. 5% Ferric chloride solution test	_
	b. Reaction with lead acetate	_
	c. Gelatin test	_
10	**Test for Saponins**	
	a. Frothing Test	+

The phytochemical tests are done for different solvent extracts individually for the positive and negative responses to series of tests (Table 2). It was interesting to note that the ethanol fractions of *S. alternifolium* that tested negatively for the presence of sugars, starch, gums, mucilage, phenol and tannins, responded positively of other tested metabolites quite consistently. With the positive indication for the presence of proteins, amino acids, fixed oils and fats, the presence of secondary metabolites namely alkaloids, glycosides, flavonoids, phytosterols and saponins were confirmed.

Analysis of Ethanolic Extract of *Syzygium alternifolium* by Gas Chromatography- Mass Spectrometry (GC-MS)

Analysis of Ethanolic Extract of *Syzygium alternifolium* (EESA) by GC-MS showed the presence of various components such as Glycerin, Pentanoic acid, 4-oxo-, 4H-Pyran-4-one,2,3- dihydro-3,5- dihydroxy-6- methyl-,2- Furancarboxaldehyde, 5-(hydroxymethyl)-, 1-octanol, 2-butyl-,2,5-monomethylene-1-rhamnitol, 1,6 anhydro-a-D-galactofuranose, 1,6 anhydro-a-D-glucofuranose, Hexanedioic acid, bis (2-ethylhexyl) ester, 1,2-Benzene dicarboxylic acid, diisooctyl ester and Squalene (Table 3).

Table 3: Components identified in Ethanolic Extract of *Syzygium alternifolium* (EESA)

No.	RT	Name of Compound	Molecular formula	MW	Peak Area%
1.	2.33	Glycerin	$C_3H_8O_3$	92	17.92
2.	2.92	Pentanoic acid,4-oxo-	$C_5H_8O_3$	116	11.69
3.	3.89	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	9.16
4.	4.80	2-furancarboxaldehyde,5-(hydroxymethyl)-	C_6H_6 O_3	126	13.91
5.	5.69	1-Octanol,2-butyl-	$C_{12}H_{26}$ O	186	2.67
6.	7.80	2,5-monomethylene-1-rhamnitol	$C_7H_{14}O_5$	178	4.21
7.	8.33	1,6-Anhydro-a-D-glucofuranase	$C_6H_{10}O_5$	162	21.19
8.	9.61	1,6-Anhydro-a-D-galactofuranose	$C_6H_{10}O_5$	162	10.41
9.	19.29	Hexanedioicacid,bis(2-ethylhexyl)ester	$C_{22}H_{42}O_4$	370	2.90
10.	21.35	1,2-Benzenedicarboxylic acid,diisooctyl ester	$C_{24}H_{38}O_4$	390	1.96
11.	25.24	Squalene	$C_{30}H_{50}$	410	3.98

Acute toxicity studies

No abnormality was observed in the acute oral toxicity testing in rats with the single dose of 2 gram/kg of the extracts. Therefore the herbal extracts are categorized under acute toxicity class 5- > 2000 mg- 5000 mg/kg (OECD Guidelines, 2000). These findings provide information on the doses to be given in the subsequent pharmacological studies. At the tested dose extracts did not cause any of the toxic signs noted below till the period of 14 days observation after the administration of the plant extract in the tested animals. These results observed in the study is accordance with many successful and commendable recommendations made earlier. It had been shown raw drugs and plant extracts with similar properties have tuned out useful remedies for many aliments.

DISCUSSION

The extract of *S. alternifolium* revealed the presence of various phytochemicals such as alkaloids, carbohydrates, flavonoids, glycosides, phenol, resins, saponins, tannins and steroids which are summarized in table (Table 2). Ethanol extracts have been more profuse that other organic solvents as alcoholic extractions at the most polar only next to the water[15]. Flavonoids are a group of plant metabolites which provide better health benefits through each cell signaling pathways and antioxidant effects and used reduced risk of cancer, heart disease, asthma and stroke[16]. Glycosides are also like a molecule and used as atrial flutter, atrial fibrillation, paroxysmal tachycardia, congestive heart failure[17].

The drug dosage administered in the present context with *S. alternifolium* were free of negative responses in Behavioral Toxic Signs namely sedation, restlessness, drooping head, severe depression, excessive preening, gnawing paw, panting, irritability, aggressive and defensive hostility, fear, confusion, Respiratory Toxic Signs (Hypopnea, dyspnea, gasping apnea) and Ocular Toxic Signs: Mydriasis, miosis, lacrimation, ptosis, nystagmus, cycloplegia, papillary light reflex is good positive indication for consumptive usage. As no observable changes in body weight, feed intake and water intake of animals, it can be discerned *S. alternifolium* extracts have satisfied the safety limits meant for internal administration in the doses tested in this study.

The related species such as of *S. cumini* seeds have exerted significant poly phenolic content and the antioxidant properties of these compounds are frequently associated with the anti-diabetic effect of many plant species[18,19]. Since same phyto-compounds are also present in *S. alternifolium*, there is a possibility of presence of anti-diabetic and other pharmacological activity. Pre-clinical trials under way in this species; will scrutinize therapeutic potential in greater details.

CONCLUSION

Findings from this study reveal that the components available in fruit Ethanolic Extract of *Syzygium alternifolium* (EESA) can be both bioactive and safe. That the phyto-chemicals detected in fruit extracts are similar to those conferred with therapeutic action in reports presented on other plants suggest that preclinical investigations pursued at present are worth exploring. Compounds detected through GC-MS spectrum in ethanol extract of *Syzygium* fruits can be construed as an effort taken to pin down the specific compounds would elicit therapeutic activities. Observations on acute toxicity study reveals that there is a fair chance making potent and useful new drugs from the ethanolic fruit extract and could vouch the claims made on the TM practices. It may be mentioned here that our ongoing experiments on anti-diabetic and hepato-protective function on swiss albino mice animal models (not included in this report) also provides evidences to validate the reports on the medicinal uses of this species.

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