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Phytochemical Screening and Evaluation of Cytotoxicity and Acute Toxicity of Ethanolic Leaf Extract of *Cayratiaauriculata*

Lalitha Surulichamy[1], Anusha Dakshinamoorthi[2], Yogesh Kumar Murkunde[3], Viji Devaanand[4], K. Maheshkumar[5]

[1]Demonstrator - Department of Physiology, Sri Ramachandra Institute of Higher Education and Research (SRIHER) - Chennai, India; [2]Associate Professor of Pharmacology, Sri Ramachandra Institute of Higher Education and Research, Chennai, India; [3]Head-Animal facility/ Test Facility Management, CEFTE, Chennai, India; [4]Professor & HOD of Physiology –Stanely Medical College, Chennai, India; [5]Assistant Medical Officer/Lecturer Grade-II, Government Yoga and Naturopathy Medical College and Hospital, Chennai, India.

ABSTRACT

Introduction: The *Cayratiaauriculata* has been used as a folk medicine to treat various pathophysiological conditions.

Aim: In the present study, we evaluated the presence of major phytochemicals, cytotoxicity and acute toxicity effect of ethanolic extract of *Cayratiaauriculata* leaves.

Methodology: The phytochemical screening was carried out using chemical methods; gas chromatography-mass spectrometry (GC-MS) analysis was performed to identify the individual phytoconstituents present in it; cytotoxicity assay was performed in A549 cells and acute toxicity test was performed in the zebrafish model.

Results: The results of the qualitative analysis revealed the presence of flavonoids, phenols, coumarin, saponins, tannins, terpenoids, steroids and glycosides. *C. auriculata*was found to contain a significant amount of total flavonoid and phenol contents in quantitative analysis. Fifteen different phytoconstituents were expressed in GCMS analysis. In the acute toxicity test, the methanolic extract of *C.auriculata*did does not cause mortality or any clinical signs of toxicity in zebrafish to the maximum concentration of 100 mg/L.Therefore the LC50 value of extract was found to be >100 mg/L. Hence it can be considered safe.

Conclusion: This paper will help in considering *C. auriculate* or further pharmacological studies in future.

Key Words: Herbal extract, Cayratia, phytochemicals, Cytotoxicity, Acute toxicity, Zebrafish

INTRODUCTION

Medicinal plants and their derivatives have a long history of treating human diseases. Day by day, these medicinal plants draw the attention of worldwide researchers because of their lesser side effects and good compatibility with the human body .[1]The active compounds present in plants containing medicinal properties are considered herbal drugs. These active compounds are phytochemicals and also called secondary metabolites. Some of the common secondary metabolites found in plants include alkaloids, flavonoids, terpenoids, glycosides and phenolics.[2] Isolated bioactive molecules from plant serve as starting materials for drug development. [3] However, these secondary metabolites are found to be in meagre quantity in plant material. Due to this constraint, the extraction, purification and characterization of secondary metabolites become very crucial in the process of the plant-based drug discovery process. [4] Extraction is a preliminary and critical step in the process of discovery and isolation of bioactive material. Phytochemical analysis of raw plant materials is very significant to detect and quantify the phytoconstituents present in it. [5]

Cayratiaauriculata (*C. auriculata)* belongs to the Vitaceae family, class Magnoliopsida and phylum Tracheophyta. It is commonly called Jangliangoor and Amarchotioo. [6] Synonyms of*Cayratiaauriculata*are *Cyphostemmaauriculatum* (Roxb.), *Cissusauriculata*Roxb., *Vitisauriculata* (Rob.), and *Cayratiaauriculata* (Roxb.) Gamble. It has been reported to be distributed in Bangladesh, Bhutan, India, Myanmar, Thailand and Sri Lanka. *C. auriculata* is a climber with spongy stems, 5-foliate leaves, tetramerous flower and cherry-sized red fruits (Figure 1). *Cayratiaauriculata* has 2n= 24 chromo-

Corresponding Author:
Dr. Anusha Dakshinamoorthi, Associate Professor, Department of Pharmacology, Sri Ramachandra Institute of Higher Education and Research, Chennai, India.

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somes. Cayratia species has a significant role in the preparations of Ayurvedic medicines, homemade remedies, and natural pesticides as it has a good source of Phytochemicals.[6,7] *C. auriculata*has following significant medical application. It has been used to treat ulcers, cough, cold, intestinal worm, rheumatism, hydrocele, ulcer, diarrhoea and abscess. Its leaf decoction was used as a remedy for uterine disorder and fever.[7] The bark of *C. auriculata* is used to treat burns, boils, wounds and snakebite.[9-12] Apart from this, the shoot and leaves of *C. auriculata* were also used as vegetables.[7]Despite the widely reported therapeutic applications of *C. auriculata*, there is no research finding reporting its toxicity profile. Moreover, there was no literature available on the phytochemical processing of this plant species. To address these lacunae, in the present study ethanolic extract of *C.auriculata*leaveswas examined for the qualitative and quantitative phytochemical profile, identification of phytoconstituents through GCMS, cytotoxicity effect and acute toxicity effect in the zebrafish model.

MATERIALS AND METHODS:

Collection and Extraction of Plant Material:

The *C. auriculata* plant was collected in forest areas of Visakhapatnam district, Andhra Pradesh. The plant was authenticated by DrPadal, Associate Professor, Department of Botany, Andhra University, and Visakhapatnam- 530003. The leaves were washed thrice thoroughly with distilled water to remove the dirt and debris and then dried under shadow till it gets completely dried. The dried leaves were coarsely ground powdered and extracted using a soxhlet apparatus as follows. Briefly, about 20 g of dry leaf powder of *C.auriculata*was extracted with 500 ml of ethanol (Finar Ltd.) solvent. The filtered crude plant extract was concentrated using a rotary evaporator (Buchi, Switzerland). The thick extract was obtained and stored under -20°C for further analysis.

Qualitative preliminary phytochemical analysis

The preliminary phytochemical qualitative screening was carried out using an ethanolic extract of *C.auriculata*such as follows

- **Test for Saponins:**

About 5 ml of extract was shaken vigorously with 5 ml of warm distilled water in a test tube. The formation of stable foam was taken as an indication of the presence of saponins.[13]

- **Test for Glycoside:**

About 2 ml of extract was added to 2 ml of acetic and then cooled well in ice. Then Con. H_2SO_4 was added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (which is the aglycone portion of glycoside.[13]

- **Test for Coumarin:**

To 2 ml of extract 2 ml of 10% sodium hydroxide was added. The appearance of yellow colour indicates the presence of coumarin.[15]

- **Test for Alkaloids:**

To 2 ml of extract, 2 ml of the con. HCL was added. Then few drops of Mayer's reagent were added. The presence of green colour or white precipitate indicates the presence of alkaloids.[13]

- **Test for Flavonoids**

To 5 ml of extract, 3 ml of lead ethanoate solution was added. The formation of buff-coloured precipitate was taken as an indication of the presence of flavonoids.[14]

- **Test for Tannins:**

To 1 ml of extract, 2 ml of 5% ferric chloride was added. The formation of green, blue-black, or blue-green indicates the presence of tannins.[15]

- **Test for Phenols:**

To 1 ml of extract,2 ml of distilled water followed by a few drops of 10% ferric chloride was added. The formation of blue or green colour indicates the presence of phenols.[15]

- **Test for Terpenoids:**

About 2 ml of extract was dissolved in 2ml of chloroform and evaporated to dryness. 2ml of concentrated sulphuric acid was then added and heated for about 2min. Development of a greyish colour indicates the presence of terpenoids.[16]

- **Tests for steroids**

About 2 ml of extract was dissolved in 2ml of chloroform and 2ml concentrated sulphuric acid. A red colour produced in the lower chloroform layer indicates the presence of steroids.[16]

- **Tests for anthraquinones:**

About 2 ml of extract was shaken with 10 ml of benzene and then filtered. And 5 ml of the 10% ammonia solution was then added to the filtrate and thereafter shaken vigorously. The appearance of a pink, red or violet colour in the lower ammonia layer was taken as the presence of free anthraquinones.[13]

Phytochemical Quantitative Analysis

The phytochemical quantitative screening was carried out using ethanolic extract of *C.auriculata*to to estimate the number of total phenolics and flavonoids by the method of.[17]

Total phenolics content

The total phenolics content of ethanolic extract of *C.auriculata*was estimated using Folin-Ciocalteau reagent. About 20 µg of the extract was taken and made up to 1 mL with distilled water. Then 500 µL of diluted Folin's reagent and 2.5 mL of 20% sodium carbonate solution were added. The mixture was shaken well and incubated in dark for 40 min and read spectrophotometrically at 725 nm. A calibration curve of gallic acid was constructed. The results were compared with the gallic acid calibration curve and the total phenolic content of the sample was expressed as mg of gallic acid equivalent (mg GAE/g extract) by using the standard curve.

Total flavonoids content

About 1 mL of extract was diluted with 200 µL of distilled water followed by the addition of 150 µL of 5 % sodium nitrite solution. This mixture was incubated for 5 min and then 150 µL of 10% aluminium chloride solution was added and allowed to stand for 6 min. Then 2 mL of 4% sodium hydroxide solution was added and made up to 5 mL with distilled water. The mixture was shaken well and left it for 15 min at room temperature. The absorbance was read at 510 nm. The total flavonoids content was expressed as rutin equivalent mg RE/g extract using the standard curve.

GCMS Analysis

Gas chromatography-Mass Spectroscopy analysis was performed to identify the phytoconstituents present ethanolic extract of *C.auriculata*leaves. It is an effective chemical analysis and also a common confirmation test.[18] A Shimadzu GC-2010 Plus gas chromatograph was used for analysis. The sample was introduced by split injection of ratio 10:1. The oven temperature was programmed to increase as follow, 35°C for 2 minutes, then rise by 20°C per minute to reach 450°C and remain at 450°Cfor 5 minutes. The helium is used as carrier gas at a flow rate of 2 ml/minute. The software GCMS solution ver. 2.6 was used for analyses. Identification of the components present in the extract was determined by comparing the name, molecular weight, and structure of the spectrum of known components stored in the library of National Institute Standard and Technology (NIST) library V which was provided by the instrument software.

Acute toxicity study in zebrafish

60 adult zebrafish of both male & female with a mean body length and weight of 3±0.5 cm and 0.334±0.05 g respectively were procured from Whizbang Bioresearch, Chennai. The acute toxicity study was performed as per OECD 203. After acclimatization, the fish were randomly divided into six groups of 10 fishes each. The test solution was prepared by dissolving the required quantity of the test item in the aquarium habitat water of known quantity. The fishes in Group I, II, III, IV and V was treated with extract at different concentrations during the test period of 96 hours, that is, 100, 50, 25, 12.5, and 6.25 mg/L respectively. The fishes in Group VI were normal control. The exposure solutions were maintained at optimum pH, temperature and dissolved oxygen concentration throughout the study as same as in the acclimation procedure. The test fishes were observed and recorded at 24, 48, 72 and 96 hours for mortality and morbidity. Observations were done at 0, 3, 6, 24, 48, 72 and 96 hours for clinical signs of toxicity. At the end of the test period, fishes were euthanizedinTricaine (MS-222) and subjected to necropsy.

Histology of zebrafish

For the histopathology analysis, the fish was fixed in 10% neutral buffered for 48 h. Then the fish were processed in graded concentrations of alcohol, xylene and impregnate in paraffin. Processed tissues were embedded in paraffin block and whole body sagittal sections were prepared at 5- micron thickness mounted on slides and stained with Haematoxylin and Eosin stain. The analysis of slides was performed under a light microscope [Optoscope] and photographed with the camera (digital). Slides were scored as per the method described based on the severity of histological changes [19]

RESULTS & DISCUSSION

In the present study, we established the phytochemical and toxicity profile of the ethanolic extract of *C. auriculata* leaves for the first time. No studies were carried out in this species before. Therefore, the further comparison was made on the same plant genus. As *C. auriculata* has been used as a crude extract in folk medicine, we have used the crude ethanolic extract of *C. auriculate* or all our experiments. Moreover, the crude extract will contain a mixture of bioactive compounds. Though the plant-based medications are often considered to be safe and have no side effects, [20] it is essential to derive the safety profile of the particular plant extract to determine the dose level for the examination of the therapeutic index of drugs through subsequent pharmacological studies.Hence we scrutinized the cytotoxic potential and acute toxic potential of *C. auriculata* in a zebrafish model.

Phytochemical preliminary Qualitative Analysis

The preliminary phytochemical screening using chemical methodsconducted on the ethanolic extract revealed the

presence of various phytochemicals like flavonoids, phenols, coumarin, saponins, tannins, terpenoids, steroids and glycosides. The qualitative analysis for the *C.auriculata* extract is shown in Table 1. In another study, phytochemical screening of *Cayratiapedata*(Lam.) Gagnep. var. *glabra*Gamble has been reported to contain carbohydrates, proteins, amino acids, alkaloids, anthraquinones, flavonoids, glycosides, phenols and tannins, steroids and sterols, triterpenoids and volatile oil. [21] *Cayratiatrifolia*was found to contain kaempferol, myricetin, quercetin, triterpenes and epifriedelanol, steroids, terpenoids, flavonoids, tannins, hydrocyanic acid and delphinidin. [22] The leaf and stem of *Cayratiagracilis*showed the presence of carbohydrates, tannins, saponins, flavonoids, balsams, resins, terpenes, alkaloids and sterols. [23]Ethanol extract of galing stem (*C. trifolia*Domin.) shows the presence of alkaloids, saponins, terpenoids, tannins, and flavonoids. [24] These results show that all Cayratia plants consist of the following common phytochemicals, flavonoids, glycosides, phenols, tannins, steroids, terpenoids, saponins, and alkaloids. These phytochemicals have certain pharmacological properties. For example, phenolics compound act as a reducing agent, hydrogen donor, metal chelator[25] and has anticancer and cardioprotective activity . [21] Flavonoids act as an antioxidant .[23, 24, 25] Tannins have astringent and anti-diarrhoea activity. Saponins are known to have activity against gastro-intestinal infections and cardiovascular diseases . [23]

Phytochemical Quantitative Analysis

The phytochemicals present in the extracts was quantitatively determined by standard procedure. The total phenolic and flavonoid content in the ethanolic extract of *C. auriculata* was estimated to be 111.36 mg GAE/g and 26.32 mg RE/g extract. In another study, the stem ethanolic extract of *C.trifolia*was estimated to contain total phenol, tannin, alkaloid, flavonoid and saponin contents as 34.97 ± 0.4, 54.52 ± 0.3, 33.74 ± 0.68, 26.07 ± 0.40, and 39.52 ± 0.50 mg/g respectively.[25] The ethanol extract of *C. pedata*var. *glabra*was found to contain 131.7 ± 3.6 and 52.8 ± 12.9 mg TAE/g extract of Total phenolics and Tannin respectively.[21] *C. pedate*and*C.auriculata*contain a large amount of phenolic content.

GCMS Analysis

GC-MS chromatogram analysis of the ethanolic extract of *C. auriculata* indicating the presence of fifteen different phytochemical constituents by comparing their retention times, molecular formula and molecular weight (MW) and mass spectra [Figure 2]. GC-MS analysis for biomolecules in plant extract provides deep insight into the medicinal properties of the plant. [18] The various compounds detected *b*y GC-MS analysis in *C. auriculata*is shown in (Table 2). In which, the carbonic acid was identified to have an essential role in nitrogen base protonation in blood serum. [26] Further study on predicted biomolecules will help identify the pharmacological activity of each compound.

GC-MS analysis on ethanolic extract of *C. trifolia*stem exhibited 20phytoconstituents. In which the following compounds are found to present in higher concentration, hexadecanoic acid, ethyl ester, phytol, tetratriacontane, stigmasterol, nonacosane and octadecane.[27]

Acute toxicity study of the extract in zebrafish

Zebrafish has around 70% of homologous genes to that of humans. It has become an efficient model vertebrate in toxicity and pharmacology studies .[32-34] Hence, we presumed to evaluate the acute toxic potential of *C. auriculata*in in the zebrafish model. The test conducted to determine the LC_{50} value of extract in 96 hrs. of exposure. The results show that there were no morbidity, mortality or clinical signs of toxicity were observed in all the experimental groups throughout the study. [28-31]All the test fish were found to be normal when compared to the control. There were no treatment-related gross pathological changes were visualized across different test groups in comparison with the control group. These data show that the LC_{50} of ethanolic extract of *C. auriculata*was found to be greater than 100 mg/L under the tested experimental conditions in the present study. As per Organization for Economic Co-operation and Development (OECD) and European Chemicals Bureau (ECB), the pollutants are categorized as harmful to zebrafish if., 10 mg / L < LC 50 < 100 mg / L. [35] Based on this categorization, ethanolic extract of *C. auriculata*was considered to be safe. An acute toxicity study of ethyl acetate extract of *C. trifolia* was performed as per OECD guideline No. 420 in female Wistar rats. The result reveals that *C. trifolia*was found to be safe up to the dose of 2000 mg/kg .[36]This result is in line with our study that *Cayratia*species shows no toxicity.

Histopathology of zebrafish:

Histopathological investigations were carried out to find out any changes in the cellular morphology and architecture in test fish when compared with control fish. Acute toxicity studies on different doses of *C. auriculata* leaf extracts showed no discrete histopathological changes in the gills, kidney, liver, and intestine, heart, and muscle tissue of the test fishes in comparison with control group fishes (Figure 3). There was normal cellular architecture observed in all the experimental groups.

CONCLUSION

To conclude based on the above results, it was found that the ethanolic extract of *C. auriculata* holds more phytochemicals and contains various phytoconstituents which was detected through GCMS. Future studies on these phytoconstituents may be useful in identifying their pharmacological efficacy. The extract shows a cytotoxic effect against A549

cells in comparison with control. Acute toxicity test results show that the extract is safe in testing with zebrafish. Thus *C.auriculata*canbe further studied for its pharmacological activity in future.

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Table 1: Phytochemical analysis for the ethanolic extract of C. *auriculata*.

Sl. No.	Phytochemical compounds	Inference
1	Alkaloids	Absence
2	Anthraquinone	Absence
3	Flavonoids	Presence
4	Phenols	Presence
5	Coumarin	Presence
6	Saponins	Presence
7	Tannins	Presence
8	Terpenoids	Presence
9	Steroids	Presence
10	Cardiac Glycosides	Presence

Table 2: Compounds identified in ethanolic extract of *C. auriculatain* GC-MS analysis

ID	Name of the compound	Retention Time	Molecular Formula	Molecular weight	Peak Area
1	TRANS-2,2-DIMETHYL-3-(2-METHYL-1-PROPENYL)-1- (PHENYLTHIO) CYCLOPROPANE METHENAMINE	7.058	C16H23NS	261	33744
2	CYCLOPENTA [5,6] PENTALENO[1,6A-B] OXIREN-7-OL	43.759	C11H20O2	184	3844
3	N-(T-BUTYL)-2-BENZOYLBENZAMIDE	44.175	C18H19NO2	281	4629
4	CARBONIC ACID	44.246	C18H17BrO3	360	5626
5	(3R*,1'S*,4'R*,5'R*,6'R*)-3-[6'-[(TRIMETHYLSILYL) ETHYNYL] BICYCLO [2.2.1] HEPTEN-5'-YL] CYCLOPENTANONE	44.525	C17H24OSi	272	23450
6	PYRANO[3,4-B] INDOL-3(9H)-ONE, 1-(4-PENTYNYL)- $$ 1-(PENT-4-YNYL)PYRANO[3,4-B]INDOL-3-ONE	44.529	C16H13NO2	251	36481
7	3,4,5,6-TETRACHLORO-12,12-DIMETHOXY-(ENDO, ENDO)-TETRACYCLO[6.2.1.1(3,6).0(2,7)]DODECA-4,9-DIEN-11-OL	44.508	C14H14Cl4O3	370	6494
8	CESIUM TRIMETHYL FLUORO) ALUMINATE	44.640	C3H9AlCsF	224	14293
9	1-HYDROXYETHOXY-2-METHOXYCARBONYL -4, 6-DINITRO-BENZENE	44.625	C11H13N2NaO9	340	5716
10	CHRYSENE, 1,2,3,4,4A,5,6,11,12,12A-DECAHYDRO-8-METHOXY-2-(METHOXYMETHOXY)-4A-METHYL-, (2.ALPHA.,4A. ALPHA ., 12A.BETA.) - (.+-.)- $$ 17-METHOXY-3.BETA.- METHOXYMETHOXY-D-HOMO-18-NORANDROSTA-8,13,15,17-TETRAENE	44.908	C22H30O3	342	8694
11	2,4-CYCLOHEPTADIENE-1,6-DIONE(MORPHOLINE)FE(CO)2	44.905	C13H15FeNO5	321	12846
12	2-PROPENIMIDOTHIOIC ACID	45.040	C14H20N2O3S2	328	8463
13	STYRYL HEPTAMETHYL TRIGERMANE	45.452	C15H28Ge3	430	14919
14	1-PROPANAMINE, N,2-DIMETHYL-N-N-NITROSO- $$ 1-ISOBUTYL-1-METHYL-2-OXOHYDRAZINE # $$ 1-ISOBUTYL-1-METHYL- 2-OXOHYDRAZINE $$ 1-ISOBUTYL-1-METHYL-2-OXOHYDRAZINE	45.100	C5H12N2O	116	76
15	11,18-EPOXY-18-HYDROXYIMINO ISOMER	45.539	C32H54O4	502	2140

Table 3: Cytotoxic activity of *C. auriculata* leaf extracts against A549 cell line

Concentration of extract (µg/ml)	% inhibition
Control	0.000
12.5	1.028
25	17.789
50	31.467
100	43.834
150	61.021
200	73.671

Figure 1: *Cayratiaauriculata*.

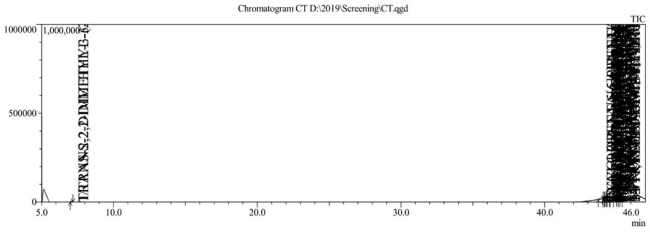

Figure 2: GC-MS spectra of ethanolic extract of *C. auriculata*.

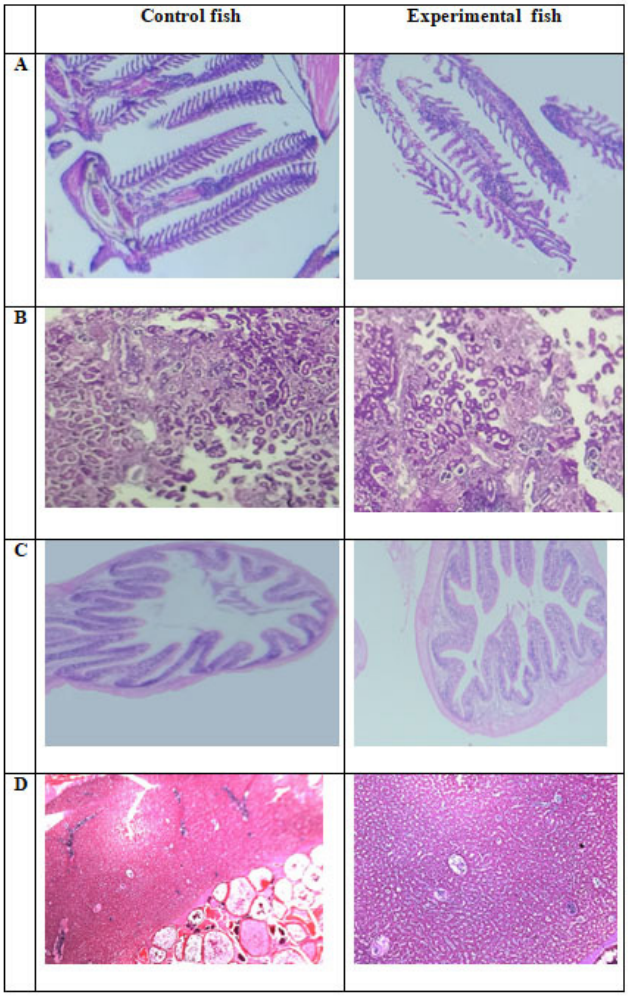

Figure 3: Histopathological changes in the gills (A), kidney (B), liver(C), and intestine (D) tissue of the experimental and control group fishes.