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Phytochemical analysis of leaf extracts of *Catharanthus roseus* (L.) G. don

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Abstract
The medicinal plant is the most exclusive source of life-saving drugs for the majority of the world's population. Plant-derived compound has played an important role in the development of several clinically useful anticancer agents. *Catharanthus roseus* (L.) G. Don is an important medicinal plant of the Apocynaceae family. Its leaves are used for cancer treatment. The present study aimed at determining the phytochemical profile of *Catharanthus roseus* (L.) G. Don by standard qualitative and quantitative methods. The extract was prepared by finely ground dried leaves and 80% methanol, followed by concentrating at low temperatures. The analysis revealed the presence of a good amount of alkaloids, saponins, phenols, and steroids in the methanolic leaf extract. Along with them other compounds are also found which includes the flavonoids, glycosides, and tannins. All these bioactive compounds that are found to be present in this plant have very potential medicinal roles and are widely used for the treatment of various ailments. It is the presence of these phytochemicals that impart the medicinal properties in the plants and increase its importance. The study just revealed a phytochemical profile, future studies could consider determining the profile in other solvents followed by purification of compounds and evaluation of their medicinal applications on animal models.

Keywords: Cancer, *Catharanthus roseus* (L.) g. don, pharmacology, phytochemicals, antimicrobial, MIC

Introduction
The medicinal plant is the most exclusive source of life-saving drugs for the majority of the world's population. They continue to be an important therapeutic aid for alleviating the ailments of human kinds. India has a rich and diverse flora of flowering medicinal plants. Plants have been used as medicines by all cultures from ancient times to the recent days. Medicinal plants play a vital role in human health care, about 80% of the world population use traditional medicine, concomitantly based on plant materials. The World Health Organization (WHO) predicts that the number of cases worldwide for diabetes is now 150 billion, which will double by the year 2025.

Plants produce several secondary metabolites including alkaloids, flavonoids, saponins, steroids cyanogenic glycosides, and terpenoids to protect themselves from the attack of naturally occurring pathogen, insects' pests and environmental stresses. The above activity of those compounds should depend on the methods and solvent used for extraction (Cragg and Newman, 2005; Sharma and Kumar, 2012) [4, 23]. Most probably herbal plants used in traditional medicine consist of a wide range of bioactive compounds that can be used as alternative therapeutic tools for the prevention or treatment of many contagious diseases. Medicinal plants are considered as clinically effective and safer alternatives to the synthetic antibiotic (Kaur and Mondal, 2014; Govindasamy and Srinivasan, 2012) [11, 7]. *Catharanthus roseus* (L.) G. Don, one of the best-studied medicinal plants, has immense medicinal value for its alkaloids. All parts of the plant including leaf, root, shoot, and stem are used for therapeutic purposes against several diseases (Van der *et al*., 2004 and Kaushik *et al*., 2017) [25, 12]. It is an evergreen herb plant growing to 1 m tall. The leaves are oval to oblong, 2.5- 9.5 cm. long and 1-3.5 cm. broad glossy green hairless with a pale midrib and a short petiole about1- 1.8 cm. long and they are arranged in the opposite pairs. The flowers are white to dark pink with a dark red center, with a basal tube about 2.5-3 cm. long and corolla about 2-5 cm. diameter with 5 petals like lobes. The fruit is a pair of follicles about 2-4 cm long and 3 mm broad (Sain and Sharma, 2013) [13]. The plant contains significant amounts of volatile compounds including caffeoylquinic acids and flavones glycosides which are known to possess antioxidant activity. It has an important role in the body defense system by acting as an antioxidant against reactive oxygen species (ROS) (Kabesh *et al*., 2015) [10]. Most reports on the anti-diabetic activity of this plant have been conducted using crude extracts (Ganga *et al*., 2012) [6] rather than the pure bioactive compounds. Periwinkle (the English name of *C. roseus*) contains dozens of alkaloids, among them vincristine and vinblastine are the major alkaloids play an important role in western medicine as potent as anticancer agents.

Vindoline and catharanthine are the major monomer alkaloids as well as biosynthetic precursors for the "dimeric" alkaloids, vinblastine (0.038 % w/w) and vincristine (0.60-0.65%), two anticancer drugs used in the treatment of acute leukemia and Hodgkin's disease. They are naturally extracted from the pink periwinkle plant. Vinca alkaloids are the second-most-used class of cancer drugs (Moudi *et al*., 2013) [15]. In India, the juice from the leaves was used to treat wasp stings. In Hawaii, the plant was boiled to make a poultice to stop bleeding. In China, it was used as an astringent, diuretic, and coughs remedies (Farnsworth, 1961) [5]. The hot water extract of the dried entire plant is taken normally by a human for cancer. Hot water extract of dried leaves is used orally to Hodgkin's disease (Virmani *et al*., 1978 [27]. The root extract is taken orally for menorrhagia (ANON, 1985) [1]. *Catharanthus roseus* (L.) G. Don contains significant amounts of volatile and phenol

compounds including caffeoylquinic acids and flavanoid glycosides which are known to possess antioxidant activity. It has an important role in the body defense system that acts as antioxidants against reactive oxygen species (ROS), which are harmful by forming such products through normal cell aerobic respiration (Salah and Nida. 1995) [21]. The immense medicinal value of *Catharanthus roseus* (L.) G. Don has been discussed in a number of literature and publications. This plant has been mostly studied concerning its anti-cancer, anti-hypertension, and anti-diabetic properties.

Generally, phytochemicals act as poisonous agents and protect the plants against insects and herbivores. Some act as regulatory growth factors of growing plants (Conner, 2009) [3]. Hesse and Manfred, (2002) [9] documented that the alkaloids showed stimulant to CNS, anti-microbial activities, sympathomimetic, vasodilator, antihypertensive, antipyretics, antimalarial and some psychoactive drugs namely methamphetamine (Yaba), amphetamine and so on produced from isolated alkaloids (Veselovskaya and Kovalenko, 2000) [26]. The plant *Catharanthus roseus* with the highest alkaloids bestows high medicinal values. Some of the characteristics of saponins include the formation of foams in aqueous solutions, hemolytic activity, cholesterol-binding properties, and hemolytic activity, and bitterness (Okwu, 2004) [18].

Material and Methodology

Collection of sample

The matured leaves of *Catharanthus roseus* (L.) G. Don were collected from Chapra city, Bihar. During the flowering and fruiting period. The collected leaves were washed under tap water to remove dust and soil particles. These leaves were kept for shade drying and then were grounded into fine powder by mechanical blender and stored in airtight containers. For the phytochemical analysis, only fresh and tender leaves were selected.

Extract preparation

For the preparation of extract, 50 gm of the dried leaf powder was mixed with 80% of 100ml Methanol in an airtight reagent bottle. The content was shaken well by keeping the bottle on the Rotator shaker maintained at 120rpm at RT for 48 hours. After this period the extract was filtered through Whatman no. 1 filter paper. The filtrate collected was concentrated by heating it at low temperatures for a certain period to obtain a viscous extract sample.

Phytochemical Analysis

The concentrated extract obtained from above was now used for the qualitative analysis of the phytochemical compounds in it by standard procedures. The analysis was carried out with a small fraction of extract.inThe analysis of each bioactive compound was done by different test for the same as follows;

1. Test for phenolics(Martinez and Valencia, 2003) [14]

a. Phenol test

To 0.5 ml of the extract, an equal volume of ferric chloride solution was added and observed for the formation of bluish black color indicating the presence of phenol compounds.

b. Ellagic acid test

For this 0.5 ml of extract was treated with a few drops of both 5% (v/v) glacial acetic acids and 5% (w/v) sodium nitrate solution. The mixture was observed for the formation of muddy yellow, olive-brown, Niger brown, and deep chocolate colors, all indicating the presence of phenols

2. Test for tannins (Parekh and Chanda, 2007) [19]

a. Gelatin test

The extract was treated with few drops of gelatin (1% w/v) prepared in sodium chloride (10% w/v) and was observed for the appearance of white precipitate indicating the presence of tannins.

3. Test for flavonoid (Parekh and Chanda, 2007) [19]

a. Flavanoid test

To 0.5 ml of extract, few magnesium turnings were added followed by a drop wise addition of conc. sulphuric acid through the sides of the test tube. The reaction mixture was observed for the formation of magenta color for flavanoid, scarlet color for flavones, and deep cherry color for the presence of flavanoid.

b. Ferric chloride Test

For this neutral ferric chloride solution was added drop wise to 0.5 ml of extract and was observed for the formation of blackish-green color indicating the presence of flavanoid in them.

4. Test for Alkaloids (Ogunyemi, 1979) [16]

a. Mayer's reagent test

To 0.5 ml of extract, 2 ml of Mayer's reagent and 1 ml of dilute hydrochloric acid were added and observed for the formation of yellow precipitation indicating the presence of alkaloids in the extract.

b. Wagner's reagent test

The extract was treated with 2 ml of Wagner's reagent and 1ml of dilute hydrochloric acid, mixed well, and observed the formation of white precipitation for the presence of alkaloids.

5. Test for steroids (Savithramma*et al.*, 2011) [22]

a. Salkowski test

To 0.5 ml of extract few drops of salkowski reagent was added followed by drop wise addition of sulphuric acid to it and observed for the formation of wine-red color for the presence of steroids.

b. Liebermann-Burchard's test

To 0.5 ml the extract, a few drops of acetic anhydride was added and mixed well followed by 1ml of conc. sulphuric acid dropped from the sides of the test tube. Then observed for the formation of the red ring at the junction of two layers for the presence of steroids.

6. Test for glycosides (Trease and Evans, 1989)

a. Keller-Killani test

The extract was treated with a few drops of glacial acetic acid for a minute and cooled. To this, 2-4 drops of ferric chloride solution was added. The content was transferred to another test tube containing conc. sulphuric acid and observed for the formation of the reddish ring at the junction of two layers indicating for the presence of glycosides.

b. Molischs' test

To 0.5 ml of the extract, 1 ml of Molisch's reagent was added, mixed well, and then 1ml of conc. sulphuric acid was dropped through the sides of the test tube and observed for the formation of the reddish-violet ring at the junction of two layers for the presence of glycosides.

7. Test for Triterpenes (Harborne, 1973) [8]

a. Salkowski test

To the extract, a few drops of concentrated sulphuric acid were added and observed the appearance of a golden yellow color to the lower layer for the presence of triterpenes.

b. Liebermann-Burchard's test

To 0.5 ml of extract, a few drops of acetic anhydride were added and mixed well. Then 1ml of conc. sulphuric acid was dropped from the sides of the test tube and observed the formation of the red ring at the junction of two layers for the presence of triterpenes.

8. Test for reducing sugar

To 0.5ml of extract solution, 1ml of water and 5-8 drops of Fehling's solution was added to the test tube hot and observed for brick red precipitate.

9. Test for Total Carbohydrate

To determine qualitatively presence of Carbohydrate 2 ml of extracts was supplemented with 1ml Benedict's reagent change in color towards brownish red depicts the presence of carbohydrate.

Quantification of Phytochemical

1. Determination of Alkaloids

Exactly 200ml of 10% acetic acid in Methanol was added to each powder sample (3.0 g) of the plant in a 250ml beaker and allowed to stand for 4 hours. The extract was concentrated on a water bath to one-quarter of the original volume followed by the addition of 15 drops of concentrated ammonium hydroxide drop-wise to the extract until the precipitation was complete immediately after filtration. After 3 hours of mixture sedimentation, the supernatant was discarded and the precipitates were washed with 20mlof 0.1M of ammonium hydroxide and then filtered using Gem filter paper (12.5 cm). The residue was dried in an oven and using electronic weighing balance the weight was determined and the percentage of the alkaloid is expressed mathematically as;

% Alkaloid = Weight of alkaloid / Weight of sample × 100.

2. Determination of Saponin

Exactly 100ml of 20% aqueous ethanol was added to 5 grams of each leaf powder sample in a 250mlconical flask. The mixture was heated over a hot water bath for 4 hours with continuous stirring at a temperature of 55°C. The residue of the mixture was re-extracted with another 100ml of 20% aqueous ethanol after filtration and heated for 4 hours at a constant temperature of 55°C with constant stirring. The combined extract was evaporated to 40ml over a water bath at 90º C. 20 cm3 of diethyl ether was added to the concentrate in a 250mlseparator funnel and vigorously agitated from which the aqueous layer was recovered while the ether layer was discarded. This purification process was repeated twice. 60mlof n-butanol was added and extracted twice with 10mlof 5% sodium chloride. After discarding the sodium chloride layer the remaining solution was heated in a water bath for 30 minutes, after which the solution was transferred into a crucible and was dried in an oven to a constant weight. The saponin content was calculated as a percentage:

% Saponin = Weight of saponin / Weight of sample × 100.

3. Determination of Phenols

gher using a sox let apparatus. The defatted sample (0.50 g) was boiled for 15 minutes with 50mlof ether for the extraction of the phenolic components. Exactly 10mlof distilled water, 2mlof 0.1N ammonium hydroxide solution, and 5ml of concentrated amyl alcohol were also added to 5ml of the extract and left to react for 30 minutes for color development. The optical density was measured at 505 nm. 0.20 g of tannic acid was dissolving in distilled water and diluted to 200 mL mark (1mg/cm3) in preparation for the phenol standard curve. Varying concentrations (0.1–1.0mg/ml) of the standard tannic acid solution were pipette into five different test tubes to which 2ml of NH3OH, 5mlof amyl alcohol, and 10ml of water were added. The solution was made up to 100ml volume and left to react for 30 minutes for color development. The optical density was determined at 505 nm. The concentration of phenol in the sample was calculated from the standard curve in mg/ml of the sample.

4. Quantitative Estimation of Steroids

1ml of test extract of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). Theergosterol was used as the standard steroid for the formation of the standard curve. Different concentration of it was used and given the same treatment as the sample. The mixture was heated in a water-bath maintained at 70±20C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank containing everything except the plant extract.

Antibacterial Activity

The extract after analysis was checked for its antibacterial activity agaisnt some of the potent bacterial pathogens. The extract was analysed for its antibacterial potential by disc diffusion method (Gulluce *et al*., 2007). The growth of test organism was maintained by sub culturing on nutrient broth and incubating overnight at 35º C. The bacterial pathogens include, *S. aureus, E. coli. Klebsiella spp., Pseudomonas aeruginosa, Salmonella spp.* Gentamycin was used as positive control whereas methanol without plant extract was used as negative control. Methanol extract containing discs (5 mm in diameter) were placed on spread culture of bacterial agar plate subsequently plates were kept in an incubator at 35º C for incubation for least 24 hours. After incubation the plates were observed for the clear zone around the disc and the diameter of zone was measured in mm called as zone of inhibition.

Results

After the concentrated extract of the leaves was ready, the first effort was made to screen the extract for the presence of

secondary metabolites also called the phytochemical compounds. The qualitative analysis was done by standard protocol and for each compound; two different tests were performed just to support the confirmed presence of the compound in the extract.

The extract was tested for the presence of phenols, flavanoid, tannins, alkaloids, steroids, glycosides, and triterpenes. Among all the phytochemicals analyzed, the extract showed negative only for the presence of triterpenes and positive for rest. The results of qualitative analysis are summarized in table no. 1 where + indicates presence and – indicates the absence of that compound. After the qualitative analysis, the extracts were now taken forward for the quantification of some of the major phytochemical compounds like Alkaloids, Saponins, Phenols, and Steroids. The quantification process was undertaken based on standard procedures and also included the use of standard compounds for drawing the standard plot. The results for the quantitative analysis of methanol leaf extract are summarized in table no. 2. The analysis revealed that plant Catharanthus roseus (L.) G. Don is a good reservoir of some of the very potential compounds that are far better than the synthetic drugs in treatments of various ailments.

These compounds are versatile in their medicinal properties and are hence widely accepted and employed in the manufacture of herbal drugs. After their analysis the extract was now asessed for its antibacterial potential against five potent pathogenic bacteria. The antibacterial potential of extract was determined as the Zone of inhibition it produced against the pathogen in correspondence to the positive control antibiotic (Gentamycin). The Zone of inhibition is measured in mm and the result for it is given in table no 3. The extract was most effective against *E. coli* and Klebsiella *spp.* and least against *P.* aeruginosa. But the overall potential of extract was effective against the bacterial pathogens.

Tables

Table 1: showing the summarized result of Qualitative analysis of phytochemicals in the methanolic leaf extract

S. No.	Phytochemical	Test	Result
1	Phenols	Phenol test	+
		Ellagic acid test	+
2	Tannins	Gelatin test	+
3	Flavonoids	Flavanoid test	+
		Ferric chloride Test	+
4	Alkaloids	Mayer's reagent test	+
		Wagner's reagent test	+
5	Carbohydrate	Benedict Test	+
6	Reducing Sugar	Fehling's Test	+
7	Steroids	Salkowski test	+
		Liebermann-Burchard's test	+
8	Glycosides	Keller-Killani test	+
		Molischs' test	+
9	Triterpenes	Salkowski test	-
		Liebermann-Burchard's test	-

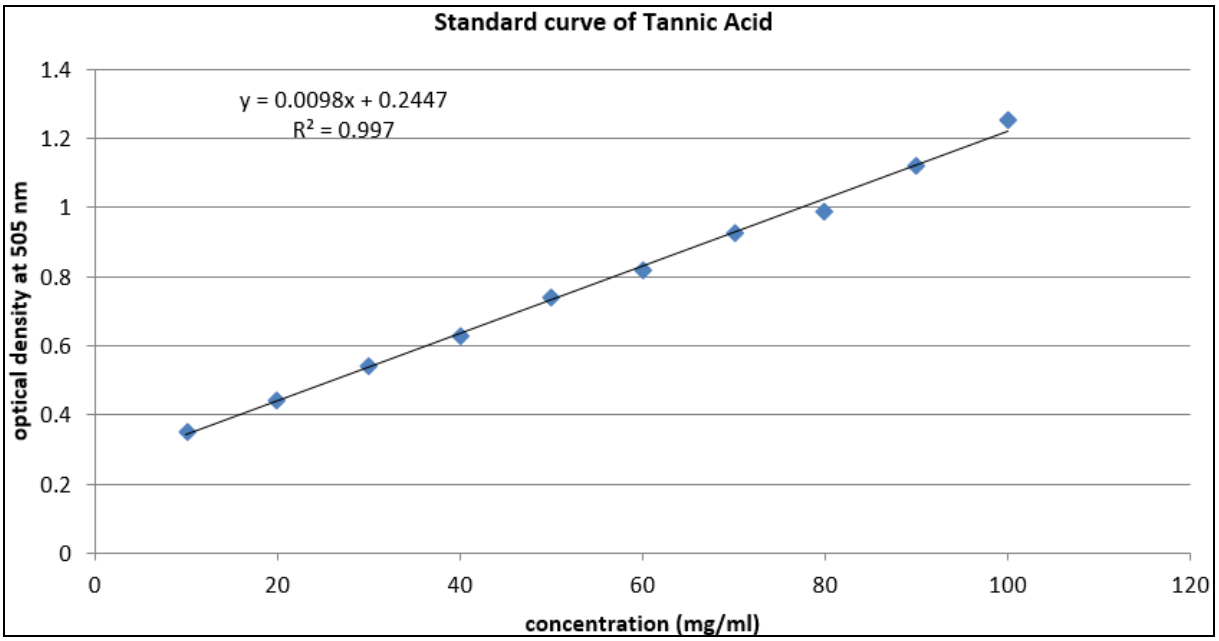
Table 2: showing the summarized result of Quantification of phytochemical in the methanolic leaf extract

S. No.	Compound name	*Catharonthus roseus* (L.) G. Don
1	Alkaloids	15.7%
2	Saponins	1.5%
3	Phenols	3.60 mg/ml
4	Steroids	5.51 mg/ml

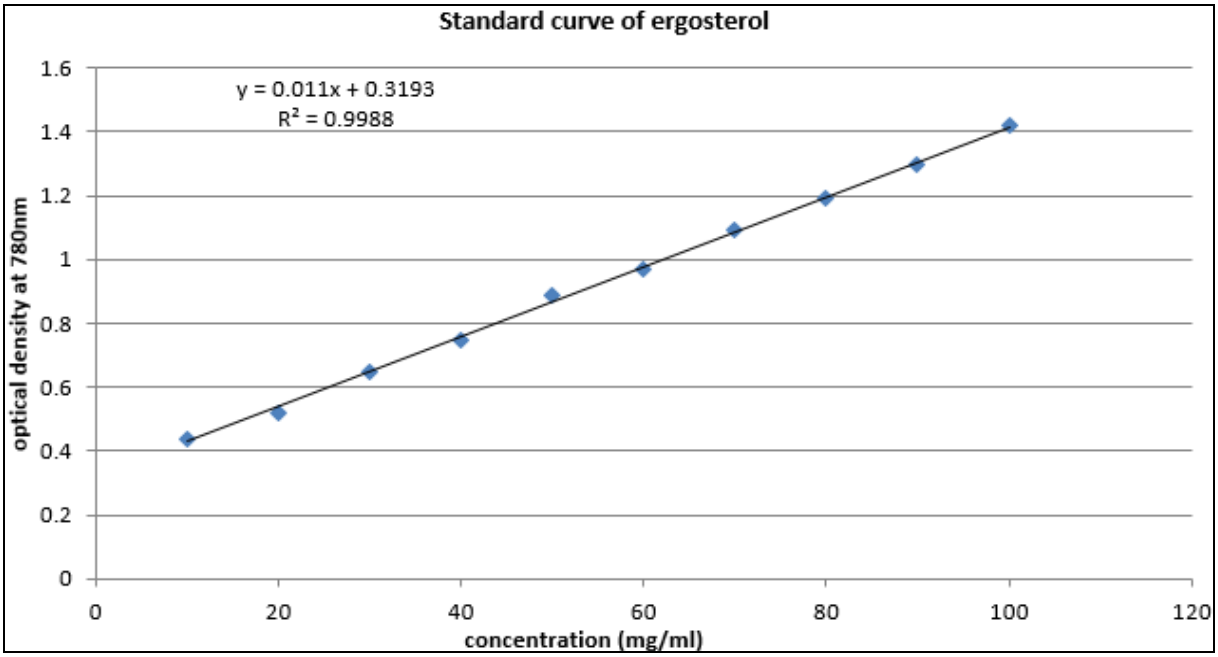
Table 3: showing the result of Antibacterial activity of methanol extract of *C. roseus*

Sample Name	Zone of Inhibition in mm				
	S. aureus	*P. aerugenosa*	*E. coli*	*Salmonella spp.*	*Klebsiella spp.*
Methanol extract	10	9	15	13	15
Gentamycin	20	15	20	18	21

Graphs



Graph 1: Graph showing the standard curve of Tannic acid for the quantification of phenols in extract



Graph 2: Graph showing the standard curve of Ergo sterol for the quantification of steroids in extract

Discussion

The phytochmeical analysis showed the presence of alkaloids, phenols, tannins and saponins in the methanol extract, the findings are similar with the result of Kabesh *et al*., 2015 [10] and Kumar *et al*., 2013 [13]. The presence of flavonoids, carbohydrates and steroids in methanolic extract is reported by Aziz *et al*., 2014 [2]. Both qualitative and quantitative study has revealed the presence of a good amount of phenolic compound in the extract driving the attention towards its potential nature. The existence of phenol compounds in this plant signpost that the selected plant may be used as a medicine. The health benefits associated with polyphenol containing preparation consumption have also been corroborated in animal studies of cancer chemoprevention; hypercholesterolemia, atherosclerosis, Parkinson's disease, Alzheimer's disease, and other aging-related disorders (Zaveri, 2006) [28]. Tannins have astringent properties, accelerate the healing of wounds, and inflamed mucous membranes. Tannins are also testified to have various physiological effects like anti-parasitic anti-irritant, antiscretolytic, and anti-microbial activities. Plants containing tannin are used to treat non-specific diarrhea and inflammation of the mouth (Ojewole, 2005) [17]. The presence of tannins in the extract is revealed under the study by qualitative tests.

Conclusion

In the present study leaf sample of *Catharanthus roseus* was taken and from it, the methanolic extract was prepared. Qualitative and quantitative analysis of the leaf extract was carried out to reveal its medicinal potential. Some potential bioactive compounds like alkaloids, flavanoid, steroids, tannins and glycosides were found to occur in leaf extract which reveals its medicinal potentiality very well. For being a potential source of medicinally important compound this plant should be considered more and further studies should be focused on purifying and determining the molecular structure of these bioactive compounds.

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