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ASSESSMENT OF NUTRITIONAL RELEVANCE OF *DIPLAZIUM ESCULENTUM* BY QUALITATIVE PHYTOCHEMICAL AND OVERALL PROTEIN PROFILING

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ABSTRACT

Diplazium esculentum is one of the very well-liked edible ferns, a common pteridophyte. Very few reports are published about the plant from India. Pteridophytes are acquainted for their biological and medicinal properties for long time but very fewevidences are seen about wetland pteridophytes. The present work focuses on assessment of nutritional relevance of *D. esculentum* collected from Alappuzha District of Kerala in India, by qualitative phytochemical and overall protein profiling. Chloroform and methanolic extracts of leaves of *D. esculentum* are used for both assays. The results showed the presence of vital phytoconstituents on qualitative analyses that included Alkaloids, Saponins, Flavonoids, Steroids, Phenols, Cardiac glycosides and Carbohydrates. On protein profiling by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE), 5 distinct bands corresponding to molecular weights ~250 kDa, ~130 kDa, ~100 KDa, ~55 KDa and ~15 KDa which showed nutritional importance of the fern, *Diplazium esculentum.* The future perspectives of study may include the quantitative estimation of phytoconstituents and proteins that paves the way for the incredible health benefits of the fern for humans.
Keywords: *Diplazium esculentum*, Leaf extract, Phytochemical, Protein profiling, SDS PAGE.

INTRODUCTION

Ferns are one among the ancient land plant groups on the surface of our earth. Contrasted with the other groups, fern plants are generally disregarded by researchers, but those are vital for the beauty and the economic uses (Bandyopadhyay and Mukherjee, 2014). *Diplazium esculentum* belonging to *Athyriaceae* family known as edible fern is a forest plant that has been used by many people since the past as a vegetable. In addition, this plant also has potential of economic, cultural,ecological and health roles(Zakiyyah and Sumardjo, 2016). *D. esculentum* is commonly found growing in humid places such as along the riverbank and also in the forest (Chandra and Srivastava, 2003). Taxonomy of *D. esculentum*is elucidated and belongs to Kingdom Plantae, Division Tracheophyta, Class Polypodiopsida, Order Polypodiales, Family Athyriaceae, Genus Diplazium, and Species esculentum (*Diplazium esculentum* (Retz.) Sw in GBIF Secretariat, 2017; USDA, 2018). This plant generally comprises of of creeping and branched rhizome; scales brown, lanceolate and around 7 to 15 mm in length, long fern leaves grow up to 100 cm and a width nearly 20 cm, quite dimorphic, raised fertile leaves, erect, sterile curved. The stalk is usually visible as green and slightly smooth, with a length of 20 - 50 cm. The leaves pinnules are lancet shaped with a length approximately 2 - 5 cm and are coarsely serrated. The erect stems are appeared to be fleshy and thick with a length around 1.2 m more. Several studies reported the traditional use of fern plants as insecticides and repellent of insects and pests (Amitand Singh, 2012; Das *et al*., 2013).

Plants are the astonishing source for the unearthing of new medicinal products in drug development (Halimatussakdiah *et al*., 2015). This activity is usually in relation to the secondary metabolite compounds present in the plants, known as natural products (Dias *et al*., 2012). Secondary metabolite is a universal term used for more than 50,000diverse substances which their compounds are usually classified according to their biosynthetic pathways. Three large molecule families are dominantly considered that include phenolics, terpenoids and steroids, and alkaloids (Ramawat and Mérillon, 2008). Several studies have already reported and published works in connection with the secondary metabolite contents in *D. esculentum* (Dash *et al*., 2017; Tongco *et al*., 2014; Chawla *et al*., 2015), but fern plants from Kerala state in India have not been widely reported, even the secondary metabolite contents of Aceh fern has not been reported.

In several countries like Malaysia, *Diplazium esculentum* (Family: Athyriaceae) is one of the very popular edible ferns usually included in one of the chief ingredients in the traditional 'Ulam' (salads) preparations. The plant is sold in bundles of fresh aerial parts in the local markets and believed to be the tastiest among other ferns locally used. They are reported to contain Ascorbic acid, riboflavin, beta carotene, calcium, iron and several proteins. The aerial parts of the plant are eaten by the people to nourish their health. Conventionally, the aerial parts are used to treat fever,

dermatitis, and measles. The leaves are believed to be effective in treating pain, wounds, dysentery, diarrhea, urinary tract infections and various skin infections. Young fronds are boiled and eaten for laxative effect. Earlier reports on various pharmacological activities as reported by previous researchers include laxative, anti-inflammatory, antioxidant, anthelmintic, antimicrobial and cytotoxic activities of the plant (Kaushik *et al.,* 2011; Ravikiran *et al.,* 2012; Ullah *et al.,* 2013; Akter *et al.,* 2014).

Introductory phytochemical screening of different species of this plant has been done by Johnson *et al.* (2011). Electrophoresis is the most rapid, relatively less tedious and highly sensitive tool to analyze various properties of protein and nucleic acid. Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) has been employed to investigate inter and intra specific variation and evolutionary relationships among the plant species (Amoroso *et al*., 2014). There is no reported evidence on molecular and biochemical systematic studies on *Diplazium* species. The nutritional potential of ethnically edible fiddle heads of riparian fern *Diplazium esculentum* distributed in the Western Ghats of India has been compared with other geographic regions. Uncooked and cooked samples possess moderate quantities of proteins, lipids and carbohydrates. Among the minerals, potassium, phosphorus, magnesium, iron, zinc and copper in uncooked fiddle heads fulfil or surpass NRC-NAS standards with favourable Na-K ratio (<1). The uncooked and cooked fiddle heads obey FAO-WHO yardsticks for six essential amino acids (His, Ile, Leu, Lys, Thr and Val) with highest quantity of lysine. The contents of seven essential amino acids (Ile, Leu, Lys, Phe, Thr, Tyr and Val) in fiddleheads are comparable or higher than soybean and wheat. The uncooked as well as cooked fiddleheads possess high quantity of palmitic acid. Adequate quantities of minerals, several essential amino acids and high in vitro protein digestibility with high protein efficiency ratios of fiddleheads of *D. esculentum* support its novelty as human diet (Greeshma *et al.*, 2018).

In the present study, qualitative profiling of phytochemical constituents and overall proteins present in *Diplazium esculentum* were done to assess the nutritional quality and the extent of edibility of the fern with respect to human health.

MATERIALS AND METHODS

Sample collection

Plant samples of *Diplazium esculentum* were collected from Alappuzha District of Kerala state in India. The plant was collected by Mr. Suresh Kumar ICAR-National Bureau of Plant Genetic Resources. (NBPGR), Chirakakode, Thrissur District and is growing at his home in Kalarcode. The authors collected the plant from his house. Leaf samples were gathered and properly labelled.

Sample processing and preparation for phytochemical screening (Tongco *et al*., 2014)

(i) Processing of plant material

The plant material was thoroughly washed under running tap water followed by distilled water to remove dust and cut into small piece, dried under shade and pulverised in to fine powder using motor and pestle. The powder was kept in plastic bags away from light, heat, moisture with proper labelling till further analysis.

(ii) Preparation of plant extracts

The phytochemical extraction was performed using two polar solvents, methanol and chloroform. Methanolic plant extract was prepared by using 100 % HPLC grade methanol and 100 % HPLC grade chloroform separately as per the method explained by Mujeeb *et al.*, 2014. For preparing the respective plant extract, 4 g of finely grounded leaves and stem of the plant were taken in 100 mL conical flask. To this, 50 mL of 100 % HPLC grade methanol/chloroform was added and covered with aluminium foil. The conical flask was placed in shaker for overnight incubation at room conditions. After incubation, the extract was filtered using double layered Whatman Filter paper No.1 in to 250 mL conical flask. The volume was made up to 200 mL by adding 100 % methanol/chloroform and stored at 4^{o}C after proper labelling.

Qualitative analyses for phytoconstituents

The methanol and chloroform extracts of plant samples were analysed for the presence of the phytochemicals using standard phytochemical methods (Choudhury *et al*., 2017; Obadoni and Ochuko, 2002; Malick and Singh, 1980)

(i) Test for Alkaloids

The test used here is Dragenodff's test. The sample extract (1mL) in chloroform was treated with 0.2 ml drops of Dragenodff's reagent and visualised brown precipitation will confirm the presence of alkaloids.

(ii) Test for Saponins

1 mL plant extract was dissolved in water and shaken well. Froth formation, which lasts for a long time, shows the presence of saponins.

(iii) Test for Flavonoids

The test used for flavanoids is Shinoda Test. 10 mL of extract was dissolved in methanol and a pinch of magnesium turnings were added to this followed by a few drops of concentrated hydrochloric acid. Formation of pink, red or magenta colour showed the presence of flavanoids.

(iv) Test for Steroids

Leibermann –Butchard test is used for detection of steroids. To 1mL of extract added equal volume of chloroform and 1mL glacial acetic acid. To this solution, add few drops of concentrated sulphuric acid. Presence of green colouration confirms the presence of steroid.

(v) Test for Phenolics

To 1mL of plant extract, 2 mL of distilled water followed by addition of 1mL of 10% aqueous $FeCl_3$ solution. Deep blue or black colour detects the formation of phenol.

(vi) Test for Quinones

To 2mL of extract, a few drops of concentrated hydrochloric acid were added and the appearance of yellow precipitate indicates the presence of quinine.

(vii) Test for Cardiac glycosides

Killer Killiani test is used for detection for cardiac glycosides. To 1mL of each extract, a few drops of glacial acetic acid and ferric chloride and 3-4 drops of concentration sulphuric acid were added. The appearance of blue-green colour indicates the presence of glycosides.

(viii) Test for Carbohydrates

Molisch's test is used to detect carbohydrates. About 10 mL of the plant extract was taken and added 1 mL water and added two drops of 1% alcoholic solution of α-naphthol. After that it was added with 1 mL concentrated sulphuric acid along the sides of the test tube so that it forms a heavy layer at the bottom. Formation of deep violet colour at the junction of the two liquids indicates the presence of carbohydrates.

The extract with maximum phytoconstituents content is selected for protein profiling.

Total protein extraction from *Diplazium esculentum* (Saha and Deka, 2017; Archana *et al.,* 2012)

For extraction of total proteins from *Diplazium esculentum,* leaf samples (100mg) were grounded with pestle and mortar and resuspended in 1 mL Phosphate Buffered Saline (PBS) buffer. Samples were mixed thoroughly in buffer and kept for 1 h at 4^{o}C. The homogenate samples were centrifuged for 13000 rpm for 10 min at room temperature and the supernatant was collected. The supernatant was transferred to a fresh 1.5 mL vial and stored at 4^{o}C until use. This supernatant was used for Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) analysis to detect the variation and diversity among the samples.

(i) SDS PAGE

(a) Preparation of stock solutions

1X running buffer (TGB Tris Glycine Buffer) was prepared by dissolving 3 g Tris base (25 mM), 14.4g of Glycine (190 mM) and 1g SDS (0.1 %) in 900 ml distilled water. The pH of the solution was adjusted to 8.3 and makeup to 1L using distilled water. Acrylamide/ bisacrylamide solution (30%) was prepared by adding 14.6g of acrylamide powder and 0.4g of bisacrylamide powder in 50 ml distilled water and stored at 4^{o}C until use. Also, 50 ml each of 1.5 M Tris pH 8.8 and 1 M Tris pH 6.8 were prepared for SDS gel preparation.

(b) Preparation of stacking and resolving gels

The components include distilled water, 30% Acrylamide/Bisacrylamide solution, 1.5M Tris pH, 10% SDS, 10% freshly prepared Ammonium per sulphate (APS) and Tetramethylethylenediamine (TEMED). Resolving Gel (1mm plate) of 15% and Stacking gel (1mm plate) of 8% were prepared.

(c) Preparation of slab gels for SDS PAGE

Glass plates were cleaned with 70% ethanol and fixed by using seal gasket and clips, resolving gel solution (15%) was poured into the space between a set of glass plates (upto 2cm from the top) and layered with a small amount of methanol (200-300 µL) to prevent gel surface from air and promote fixation. Ethanol was removed after 10-20 minutes when the gel was fixed. Stacking gel was put on the resolving gel in the remaining space and comb was inserted into the stacking gel. After polymerization, the combs, plastic clips and rubber gasket were removed and the gel was processed for loading samples

(d) Sample loading and electrophoresis

Gel plates were placed in the electrophoresis apparatus carefully to prevent bubbles formation at the bottom of the gel plates. Running buffer was added to the lower tray and then the upper tray was filled with the same buffer. About 10 µL each of the samples (supernatant) and SDS PAGE loading dye was taken in a fresh vial, mixed thoroughly for 30-60 seconds and boiled for about 10 minutes inorder to denature and neutralize the charge in the proteins in the sample. After denaturation, the samples were chilled on ice and then loaded at the bottom of each well using a micropipette. Close the lid of the apparatus tightly and connect the power supply at 100 V until the dye front reached the bottom of the gel plate

(e) Visualization of proteins in the gels

To visualize the protein in the gel, the gel was placed in 40% distilled water, 10% acetic acid, and 50% methanol solution containing 0.25% Coomassie Brilliant Blue R-250 (staining solution) and incubated for 1 h at room temperature with continuous shaking. The gel was then transferred to a mixture of 40% distilled water, 10% acetic acid and 50% methanol (detaining solution) and incubated room temperature with continuous shaking until the excess dye has been removed. As a result, the proteins in the gel can be seen as deep blue bands.Thermo Scientific Page Ruler plus Prestained Protein Ladder is used as protein ladder. It is a mixture of nine blue, orange, and green-stained proteins (10 to 250 kDa) for use as size standards in protein electrophoresis (SDS-PAGE). The protein ladder is supplied in a ready-to-use format for direct loading onto gels and there is no need to heat, reduce, or add sample buffer prior to use.

(ii) Data analysis

Total number of bands obtained from the SDS PAGE gel was analysed and compared with protein ladder gel bands for protein profiling.

RESULTS AND DISCUSSION

Sample collection

The plant and leaves of *Diplazium esculentum* were collected from Alappuzha District of Kerala (Figure 1).

Fig. 1 : *Diplazium esculentum*

Sample processing and preparation for phytochemical screening

The samples were processed and appropriate extracts were prepared as per detailed in section 2.2.1 for phytoconstituent analyses.

Qualitative analyses for phytoconstituents

The results are depicted in figure 2 and table 1.

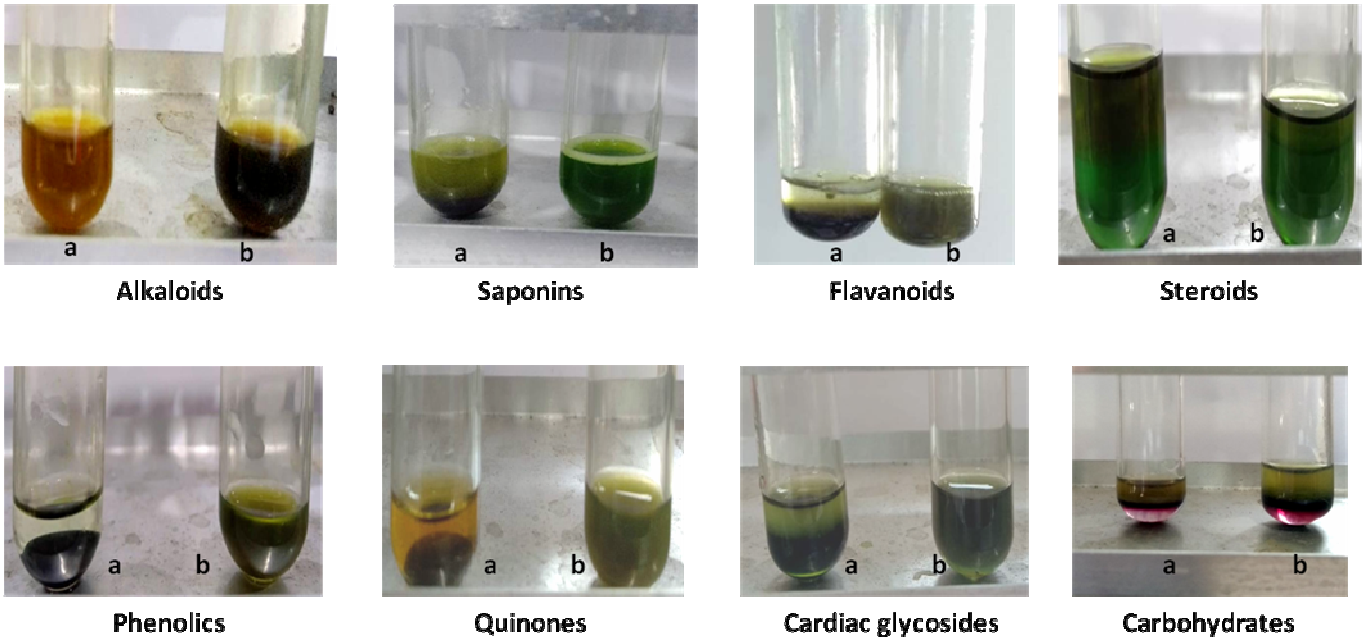

Fig. 2 : Qualitative analyses of phytoconstituents
Methanol extract of leaves of *D. esulentum* was found to have higher presence of phytoconstituents and thus selected for protein profiling.

Total protein extraction from *Diplazium esculentum*

Electrophorogram of 15% polyacrylamide gel banding pattern showing diversity of total proteins in *Diplazium esculentum*is shown in figure 3.

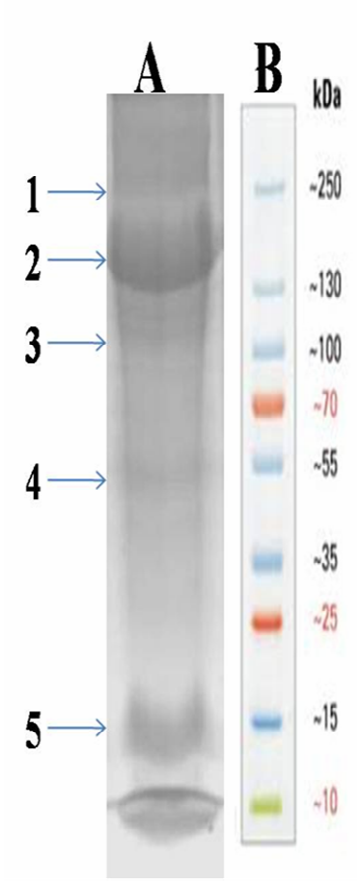

Fig. 3 : Coomassie blue staining of SDS PAGE gel (A) proteins of methanolic leaf extract of *Diplazium esculentum* (B)Thermo Scientific Page Ruler plus Prestained Protein Ladder

From figure 3, it is clear that the 5 proteins are present with molecular weights of ~250 kDa, ~130 kDa, ~100 KDa, ~55 KDa and ~15 KDa which showed their similarities towards NaDH glutamate synthase, ferredoxin dependent glutamate synthase, nitrate reductases, Ammonium transporters 1 or 2 and tuber proteins. This clearly indicated the nutritional importance of the fern, *Diplazium esculentum.*

In India, from prehistoric past we have the prosperous tradition of using plants with medicinal properties as food as well as in therapeutic diseases. Paradoxically in advanced countries, use of natural resources, use of herbal medicines is gaining importance nowadays for their fewer side effects (Roosita *et al*., 2008). Due to extensive range of geographic expansion, India is rich in its biodiversity in that way providing an immense source of plants with different therapeutic activities waiting to be explored. Wet lands are the major areas with immense biodiversity and are yet not properly evaluated for therapeutic plant resources. Similarly, pteridophytes growing in wet land have attained very less attention of scientific community. On the other hand, in recent years, many workers have explored the biological and medicinal properties of pteridophytes.

Plant procured secondary metabolites have lately become of great interest owing to their versatile applications. The phytochemical screening for biologically active secondary metabolites unveiled the presence of various phytochemicals in the leaves of *D. esculentum*. Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have reported to have anti-inflammatory effects. Glycosides, flavonoids, tannins and alkaloids are found to havehypoglycemic activities. Saponins have been accounted to possess hypocholesterolemic and anti-diabetic properties. It also exhibit medicinal properties such as anti-inflammatory, antibacterial, antifungal, antiviral, insecticidal, anticancer etc. Numerous studies have confirmed that saponins possess the unique property of precipitating and coagulating red blood cells (Okwu, 2004).

The steroids and triterpenoids have been shown to have analgesic and anti- inflammatory properties and steroids are responsible for cholesterol-reducing properties and also help in regulating the immune response (Sodipo *et al.,* 2000). Chloroform leaf extract of *Diplazium esculentum* showed the presence of alkaloid, steroid, phenol, quinine, cardioglycosides and carbohydrate whereas the methanolic leaf extract showed the presence of alkaloid, saponin, phenol, steroid, cardioglycosides and carbohydrate. Previous phytochemical studies on *Diplazium esculentum* revealed the presence of phenols, flavonoids and saponins as the main constituents. Phenols which are aromatic ring structured compounds play important role in biological as well as pharmacological studies. (Saikia and Upadhyaya, 2011). Flavonoids are known as effectual scavengers of most types of oxidizing molecules due to their hydrogen-donating ability. Flavonoids are polar compounds, so are easily soluble in polar solvents (Zannah, 2017).They are commonly found in fruits, vegetables, and plants, which normally act as antioxidants and antibacterial. Anti-bacterial activity is connected with the structure of flavonoids. Flavonoid compounds also present in the chloroform and methanol extract of *Diplazium esculentum*, and potential are reported as an anti-bacterial compound that can inhibit the growth of *Sarcinalutea, Salmonella typhimurium, Bacillus subtilis, Klebsiella pneumonia, Shigella boydii, Escherichia coli, Staphylococcus aureus and Vibrio cholera* (Akter *et al*., 2014).

The nutritional composition of *Diplazium esculentum* (known as "pako"), was determined to ascertain its use as an ingredient in culinary dishes in different parts of the world. Pako, also known as fiddlehead fern, is patronized as an ingredient in vegetable dishes and salads (Tongco *et.al.,* 2014). Moreover, due to the lack of scientific knowledge, this green leafy vegetable is mostly wasted and is a great loss in terms of food security and nutrition (Dash *et al*., 2017). The aim of this study was to extract leaf proteins and visualize the total protein content on SDS PAGE gel. It was clearly found to have a set of five protein gel bands indicating the nutritional significance of the plant.

CONCLUSION

The study indicates that the above medicinal plants gives a basis of application in traditional medicinal and the bioactivity of phytochemicals constituents was more valuable. Qualitative analysis of phytochemicals was more interesting area and also had an important application in pharmaceutical industries. The analyses were very useful for finding the presence of various chemical compounds and their quantitative estimation is needed in future for locating the world of pharmacy. The present study on the medicinal plant, *Diplazium esculentum* gives a basis of its use in medicine and develop to further drugs in pharmaceutical area. Electrophoretic analysis of total proteins is a method to investigate and to classify varieties among different species. Furthermore, analysis of SDS PAGE is simple and inexpensive, which aids in its use as a strong tool for studying the genetic diversity among different species and also evaluation of taxonomic and genetic associations at generic, specific and intraspecific levels, in addition to morphological characters. In the present study, SDS Polyacrylamide gel electrophoresis (SDS PAGE) analysis was used to analyse the total protein profile of a plant species namely *Diplazium esculentum* In the study, leaves of *Diplazium esculentum*were collected from Alappuzha district of Kerala. Total protein was extracted from the leaves of these samples and analysed through SDS PAGE. The results of the SDS PAGE analysis show that there are 5 major bands present in the total protein extract of this sample which are of sizes ~250 kDa, ~130 kDa, ~100 KDa, ~55 KDa and ~15 KDa which showed their similarities towards NaDH glutamate synthase, ferredoxin dependent glutamate synthase, nitrate reductases, Ammonium transporters 1 or 2 and tuber proteins in plants. Furthermore identification of each band is necessary since these proteins may be phytochemicals or alkaloids or antimicrobials which can be very useful in the field of medicine. So, further investigation is necessary to confirm the presence of these compounds can be confirmed through the MALDI-TOF protein sequencing of the protein bands obtained.

Table 1 : Qualitative analyses of phyto constituents in chloroform and methanol extracts of leaf samples of *Diplazium esculentum*

Phytochemical tests	Chloroform extract	Methanol extract
	Leaf	Leaf
Alkaloid	+	++
Saponin	-	++
Flavonoid	-	+
Steroid	++	++
Phenol	+	+
Quinone	+	-
Cardiac glycosides	+	++
Carbohydrates	++	++

+ Presence, ++ Strong presence, – Absence

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Conflict of Interest

The authors whose names are listed certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non financial interest in the subject matter or materials discussed in this manuscript.

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