ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *AYAPANA TRIPLINERVIS* (VAHL) R.M. KING & H.ROB

Abstract

Ayapana triplinervis(Vahl) R.M.King & H. Rob is a tropical herb which belongs to the family Asteraceae. The present investigation is focused on the analysis of phytochemical constituents, antioxidant and antimicrobial activities of the medicinal plant, *A. triplinervis*. In the present study methanol extract of leaves of *A. triplinervis* was subjected to preliminary phytochemical screening. Here the plant showed the presence of carbohydrate, proteins, phenols, flavonoids, tannins and oils. The total phenol and flavonoid contents were also analysed. The results showed that the fresh leaves of *A. triplinervis* had good levels of flavonoids and phenol. Antioxidant activity of the methanol extract was determined using DPPH radical scavenging assay and reducing power assay. The antioxidant activity increases as the concentration of extract increases. The plant extract showed antibacterial activity against *Bacillus subtilis* and *Vibrio cholerae*.

Key words: *Ayapana triplinervis*,phytochemical constituents, Antioxidant activity, Antibacterial activity.

Introduction

Ayapana triplinervis (Vahl) R.M. King & H.Rob is a tropical herb which belongs to the family Asteraceae. It is a perennial herb, which has a reddish long slender stem with long slender leaves which are aromatic. The plant has analgesic, anticoagulant, anti-helminthic, sedative, antifungal and antibacterial properties(Bose *et al*., 2007; Gupta *et al*., 2002). It was also reported that the plant has a wide range of pharmacological properties, including antioxidant, anti-inflammatory, hepatoprotective, antinociceptive and anti-ulcerous properties. It can be used for the treatment of haemorrhage, wounds, ulcers, and snakebite injuries (Shaji *etal*., 2024).The medicinal properties of the herbs are due to the presence of certain active components in the plants, which are called as phytochemicals. Alkaloids, terpenes, flavonoids, phenolics and glycosides are some of the commonly found phytochemicals and the presence or absence of these in varying concentrations, form the basis of the medicinal ability of the plants (Velu et al., 2018). Hence the aim of the present

study is to analyse the phytochemical constituents, antioxidant and antimicrobial activities of the plant, *Ayapana triplinervis*.

Materials and methods

Preparation of the plant extract

The whole plant material of *A. triplinervis*was collected fromThiruvananthapuram district of Kerala and used for the study. The leaves of *A. triplinervis*were collected from healthy plants and was shade dried and powdered in an electric mixer grinder. The dried sample was extracted using a Soxhlet apparatus. 30g powdered leaves were extracted using methanol as solvent. The condensed vapors come in contact with the sample powder and the soluble part in the powder get mixed with the solvent. After the 48 hours, the solvent collected, filtered and allowed to evaporate. This extract was used for further phytochemical analysis.

Preliminary qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out to detect the presence of various phytoconstituents such as carbohydrates, proteins,alkaloids, flavonoids, tannins, phenolics, aminoacids and oils based on standard procedures.

Test for carbohydrate

0.5 ml of extract mixed with 0.5 ml Benedict reagent and placed in a boiled water bath for 2-3min. The reddishbrown precipitate formed indicated the presence of the carbohydrates.

Test for protein

2ml of the extract mixed with 2ml of Million's reagent, white precipitate appeared which indicates the presence of proteins.

Test for alkaloids

2ml of the extract was mixed with 2-5 drops of Mayer's reagent, cream white precipitate indicates the presence of alkaloids.

Test for amino acid

2ml of extract was mixed with few drops of Ninhydrin solution. Presence of purple colour indicates the presence of amino acid.

Test for flavonoids

Add few fragments of magnesium ribbon to 2ml of extract and then add 1 ml of concentrated HCl, pink or red colour indicates the presence of flavonoids.

Test for tannin

1ml of the extract was stirred with 10ml of distilled water and filtered. A few drops of 5% $FeCl_3$were added to the filtrate. The formation of blue - green color precipitate was the indication of presence of Tannins.

Test for phenolics

3ml of 10% lead acetate solution was added to 1mL of extract. Appearance of bulky white precipitate confirmed the presence of phenolic compounds.

Quantitative analysis of phytochemical constituents

Determination of total phenol content

The total phenolic content was determined according to the method of Singleton and Rossi (1965). 0.2 ml of the leaf extract was transferred in tube containing 1.0 ml 10 % Folin - Ciocalteu reagent. After 10 min, 0.8 ml of sodium carbonate solution (7.5 % w / v) was added to the sample. The tubes were then allowed to stand at room temperature for 30 min and absorbance was read at 743 nm. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 5-50 µg/ml. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g of dry extract.

Determination of flavonoid content

The total flavonoid content was determined according to the Aluminium chloride colorimetric method of Chang et al (2002) with slight modification.The methanolic plant extract (2 ml) was mixed with 0.1 mL of 10 % Aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of deionized water. After 30 minutes of incubation at room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm.

Estimation of antioxidant activity

DPPH radical scavenging activity

The methanol extract of *A. triplinervis* was subjected to 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Free radical scavenging activity using DPPH radical scavenging activity of extract was determined according to Blois method (Blois,1958) with some modification. Different concentrations of the extract(20,40,60,80,100 µg/ml) were taken in different test tubes. The volume was adjusted to 100 µl by adding methanol. The methanolic solution of DPPH (1ml of 0.1mM) was added to these tubes and shaken vigorously. These tubes were allowed to stand for 20 minutes. The control was prepared as without extract and methanol was used as the baseline correction. That changes in the absorbance of the sample were measured at 517 nm. Percentage of radical scavenging activity were calculated according to the following formula.

$$\text{Percentage of inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

Reducing power assay

The reducing power was based on the Fe 3+to Fe 2+ transformation in the presence of extract. Various concentration of plant extracts was mixed with 2ml of phosphate buffer (0.2 M, pH 6.6) and 2ml potassium ferricyanide (1%). The mixture was incubated at 50 ºC for 20 minutes followed by the addition of 2 ml of trichloroacetic acid (10%). The mixture was centrifuged at 3000 rpm for 10 minute to collect the upper layer of the solution. 2ml supernatant from each mixture was mixed with 2ml distilled water and 0.4 ml of 0.1 % fresh ferric chloride solution. After 10 minute the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates the high reducing power.

Antimicrobial activity

Antibacterial activity was evaluated using two bacterial strains, *Bacillus subtilis and Vibrio cholerae.* The test organisms were obtained from Microbial Type Culture Collection Centre(MTCC), Chandigarh and maintained as reference stocks at -18 to - 20º C in a freezer. The bacterial strains were sub cultured and maintained on nonselective agar slants at 2-8˚ C. These cultures were used for further analysis.

Preparation of Muller Hinton Agar (MHA)

Muller Hinton Agar (MHA) medium was used for bacterial culture. The medium was prepared according to the manufacturer's instructions and sterilized at 121 °C for 15 minutes. After sterilization, approximately 20 ml of the medium was poured into sterile Petri dishes and allowed to solidify.

Inoculum preparation

Pure culture was used as inoculum. Selected 3-4 similar colonies and transferred them in to about 5 ml of suitable broth such as Tryptone Soya Broth (TSB – Himedia M 1263). Incubate at 37 °C for 8-12 hours until light to moderate turbidity developed.

Method of Inoculation

The filter paper disc diffusion technique was applied for determining antibacterial activity. A sterile non-toxic swabmounted on a wooden applicator was dipped into the standardized inoculum and rotated firmly against the upper wall of the tube to remove the excessfluid. The entire agar surface of the plate was streaked with the swab three times, rotating the plates at a 60^{o} angle between each streaking. The inoculated plates were allowed to dry for 5-15 minutes with lid closed.

Sterile 6 mm HiMedia filter paper discs impregnated with approximately 30 µl of the test sample were applied to the agar surface using aseptic techniques. The discs were placed such that the centers were at least 24mm apart. The plates were incubated immediately at 37° C and examined after 16-18 hours or later, if required. The zone of complete inhibition was measured, and the diameter of the inhibition zone were recorded to thenearest millimeter.Discs soaked in pure solvent and dimethyl sulfoxide (DMSO) were used as negative control.

Results

Preliminary phytochemical screening

In the present study,eight phytochemical screening tests have been carried out. They are test for carbohydrates, proteins, alkaloids, amino acids,flavonoids,phenol, tannin and oil. The results are represented in table 1.When the extract treated with Magnesium ribbon and conc. HCl, the extractshowed red colour. It reveals the presence of flavonoids in the sample. The formation of white precipitate with Million's reagent indicates the presence of protein.Treatment with lead acetate gives white bulky precipitate, it indicates the presence of

tannins.When the extract treated with Benedict reagent, it shows a yellowish green colour, it indicates the presence of carbohydrates. When the extract is treated with Mayer's reagent, no yellow colour precipitate is formed. It indicates the absence of alkaloids.Whenthe extract is treated with dilute iodine solution, a transient red color appeared. It confirmed the presence of phenolics. When the extract is treated with Ninhydrin solution, there is no purple colour precipitate. It indicates the absence of amino acids. When the extract is pressed between two filter paper, oil is formed on the paper. It indicated the presence of oil.

Table1: Preliminary phytochemical screening of methanol extract of *Ayapana triplinervis*

Phytochemical constituents	Presence/Absence of the phytochemicals
Carbohydrates	+
Proteins	+
Alkaloids	–
Amino acids	–
Flavonoids	+
Phenolics	+
Tannins	+
Oils	+

'+' indicates presence and '–' indicates absence

Quantitative Analysis

The total phenol and flavonoid content of methanol extract of *A. triplinervis is* 56.66mggallicacid equivalent/g and 7.20mgcatechin equivalent/g respectively. The result indicates that fresh leaves of *A.triplinervis*is a good source of flavonoids and phenols.

Antioxidant activity

In the present study,two free radical scavenging assays are carried out to analyze the antioxidant activity ofthe leaves of *A. triplinervis*.

DPPH Radical Scavenging Assay

Antioxidant activity of the leaf extract of *A. triplinervis* was determined by DPPH assay. It is the ability of the phytochemicals in the extract to scavenge the DPPH free radical. In the DPPH method, the antioxidantsreact with stable free radicals and α,α– diphenyl –β– picrylhydrazyl (deep violet colour)converted to α,α- diphenyl –β-picrylhydrazine with discoloration. The degree ofdecolourisation indicates the radical scavenging capacity of the sample extract. In the present study, the leaf extract of*A. triplinervis* exhibited the antioxidant activity at varying concentrations (25,50,100,200,400µg/ml).The free radical scavenging activity increases with increase in concentration of the extract (Figure1).

Reducing power assay

The reducing power of the methanolic leaf extract of *A. triplinervis*at various concentrations (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) showed significant reducing power (Figure 2). The extract showed a dose dependent increase in reducing power. In reducing power assay, the antioxidants present in thesample can reduce the Fe 3+ to Fe 2+ by donating an electron.

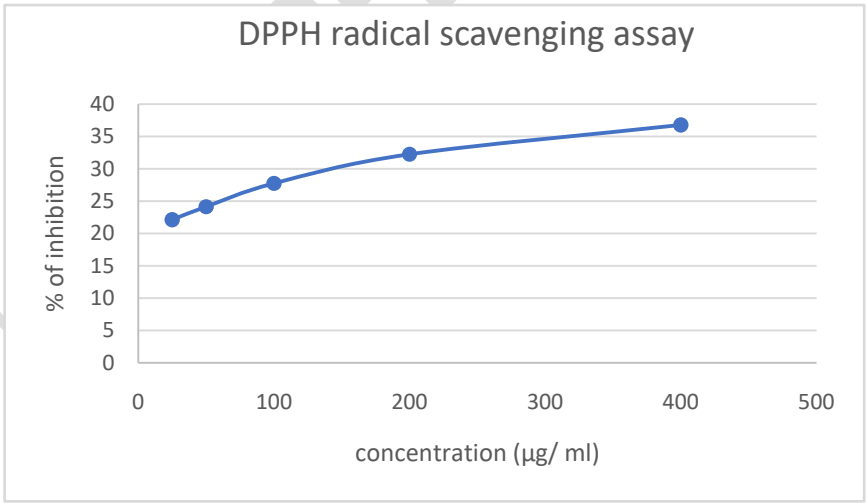

Figure 1: DPPH radical scavenging activity

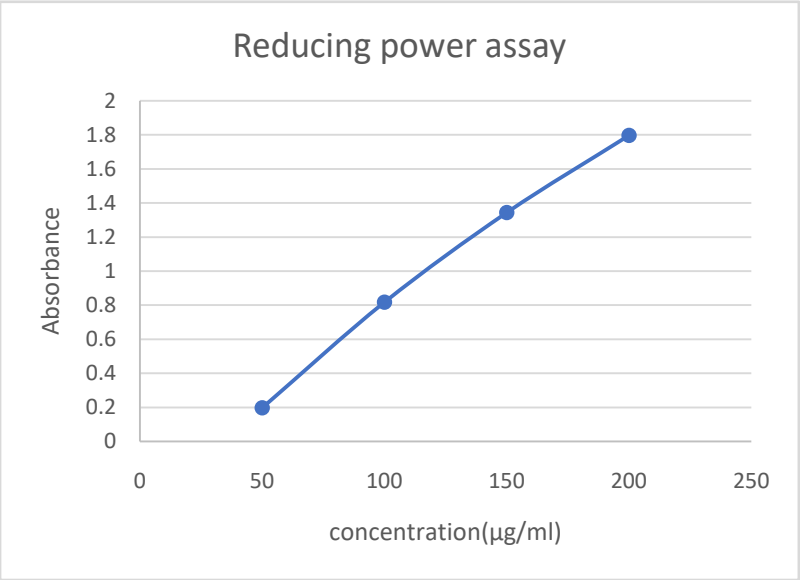

Figure2: Reducing power assay

Antimicrobial activity

Evaluation of anti- bacterial activity

The methanolic leaf extract was evaluated for antibacterial activity using the disc diffusion method. The assay was performed against two bacterial strains, *Vibrio cholera* and *Bacillussubtilis*. The antibacterial efficacy of the extract was determined by measuring the presence and diameter of the zone of inhibition around the sample-impregnated discs. The methanolic leaf extract exhibited antibacterial activity against both tested organisms. The extract showed a zone of inhibition measuring 12 mm against *Bacillus subtilis* and 9 mm against *Vibrio cholerae*. The results indicate that the extract demonstrated comparatively higher antibacterial activity against *Bacillus subtilis* than *Vibrio cholerae*.Figure 3 illustrates the antibacterial activity of the extract against *Bacillus subtilis*, while Figure 4 shows the antibacterial activity against *Vibrio cholerae*.

Figure 3: Antibacterial assay on *B. subtilis*Figure 4:Antibacterial assay on *V. cholerae*

Discussion

In the present study the methanol extract of *A. triplinervis* was subjected to phytochemical screening. Here the plant showed the presence of carbohydrate, proteins, phenols, flavonoids, tannins and oils. The earlier report about the phytochemical analysis in *A. triplinervis* showed the presence of glycosides, terpenes, phenols, flavanoids, reducing sugar, proteins, carbohydrates and alkaloids (Mamatha et al., 2017). The total phenol and flavonoids content in *A. triplinervis* were also analyzed. Then it wasfound that the total phenol content was 76.66 mg GAE/g and flavonoidcontent was 37.20 mgQUE/g. The result indicates that fresh leaves of *A. triplinervis* had highestlevels of flavonoids and phenols. The hydroxyl groups of phenolic compounds can directly contribute to antioxidant action (Bendary et al., 2013). Phenolic compounds exhibit free radical inhibition, peroxide decomposition, oxygen scavenging in biological systems and prevent oxidative disease burden (Aryal et al., 2019).

Antioxidantactivity of the fresh leaves of *A. triplinervis* was determined by DPPH assay. It is the abilityof the phytochemicals in the extract to scavenge the DPPH free radical. The molecule or compoundwith antioxidant capacity will react with a free radical DPPH and scavenge it. The DPPH assay method is based onthe reduction of DPPH (Shekhar and Anju, 2014). *A. triplinervis* exhibited the antioxidantactivity at varying concentration of the leaf extracts.The free radical scavengingactivity increases with increase in concentration of the extract. In the present study, the fresh leafsample of *A. triplinervis* showed a dose dependent reducing power activity also. The rising absorbance shows elevated antioxidant activity (Kadu et al., 2022). Extracts react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric ferrous complex (Vasyliev et al., 2020). In the present study, it was observed that the plant has good amount

of total phenol content. Earlier reports also proved that the total phenol content significantly correlated with the antioxidant activity (Chavan et al., 2013; Afify, et al., 2012).

Antibacterial activity of methanolic leaf extract of *A.triplinervis* against *Bacillus subtilis* and *Vibrio cholerae*was also evaluated. The results demonstrated that the plant extract exhibited antibacterial activity against both tested bacterial strains. The methanolic extract produced a zone of inhibition of 12 mm against *Bacillus subtilis* and 9 mm against *Vibrio cholerae*, indicating comparatively higher susceptibility of *Bacillus subtilis* to the plant extract.

The observed antibacterial activity may be attributed to the presence of bioactive secondary metabolites in the plant extract. Plant-derived secondary metabolites such as flavonoids, alkaloids, phenolics, tanninsand terpenoids are known to possess antimicrobial properties. These compounds can inhibit bacterial growth by disrupting cell wall synthesis, altering membrane permeability, or interfering with essential metabolic pathways. Similar findings have been reported in previous studies, which suggest that secondary metabolites present in plant extracts contribute significantly to antibacterial activity (Jan, 2024).

Conclusion

A. triplinervis a medicinally valuable plant in India and has been used from ancient times in traditional systems of medicine. This plant is known to possess a wide variety of medicinal valuesand this is due to the presence of various secondary metabolites. The preliminary phytochemical screening confirmed the presence ofcarbohydrates, proteins, flavonoids, phenols, tannin and oil in this plant. The total phenol and flavonoid content was estimated. The result indicates that fresh leaves of*A. triplinervis* had high level of flavonoid and phenol content.The DPPHradical scavenging assay and reducing power assay proved that the plant has antioxidant activity.The antioxidant activity increases with increasing concentration of the extract. The plant has antibacterial activity against *Bacillus subtilis* and *Vibrio cholera*.The findings of the present study suggest that *A. triplinervis* may serve as a potential source of natural antibacterial agents. However, further studies involving isolation and characterization of active compounds, determination of minimum inhibitory concentration (MIC), and in vivo validation are required to confirm its therapeutic potential

Acknowledgement

The authors are grateful to the DST – FIST and DBT- STAR schemes atPG and Research Department of Botany, S N College, Kollam for providing the facilities for research.

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