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RESEARCH ARTICLE

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

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Abstract

Ayapana triplinervis (Vahl) R.M.King and 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 Bacill us subtilis and Vibrio cholerae.

Introduction:-

Ayapana triplinervis(Vahl) R.M. King and 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). 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 evaluate the phytochemical constituents, antioxidant and antimicrobial activities of the methanolic leaf extract of Ayapana triplinervis.

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Materials and Methods:-

Preparation of the plant extract:-

The whole plant material of A.triplinerviswas collected fromThiruvananthapuram district of Kerala and used for the study. The leaves of A.triplinerviswere 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. Thereddishbrown 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.

Test for oil:-

Spot test: A small quantity of extract was pressed between two filter papers. Oil stain on the paperindicated the presence of fixed oil.

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. 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 assay:-
The methanol extract of A. triplinerviswas 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(25,50,100,200 and 400µg/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.

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

DPPH radical scavenging activity of different concentrations ofascorbic acid was also determined.

Reducing power assay:-
The reducing power was based on the Fe 3+to Fe 2+ transformation in the presence of extract. Various concentrations of plant extracts were 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 HiMedia filter paper discs (6mm) 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 were soaked in pure solvent and dimethyl sulfoxide (DMSO) were used as negative control.
The experiment was repeated thrice and the results were the mean of three replicates.

Statistical analysis:-
For statistical analysis of the quantitative data, the data obtained were subjected to one way analysis of variance (ANOVA).

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 presence and absence of these phytochemicals are represented in Table 1.The results of phytochemical screening revealed the presence of carbohydrates, proteins, flavonoids, phenolics, tannins and oils in the methanolic leaf extract of A. triplinervis.

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.triplinervisis56.6 ±0.54mg gallicacid equivalent/g and 7.43±0.33mgcatechin equivalent/g respectively. The result indicates that fresh leaves of A.triplinervisis 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 methanolic leaf extract 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). After reaction,α,α– diphenyl –β–picrylhydrazyl is 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 ofA.triplinervis exhibited the antioxidant activity at varying concentrations (25,50,100,200,400µg/ml).The free radical scavenging activity increases with increase in concentrations of both the ascorbic acid and extract (Figure1 and 2).

Reducing power assay:-

The reducing power of the methanolic leaf extract of A.triplinervisat various concentrations (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) showed significant reducing power (Figure3). 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.

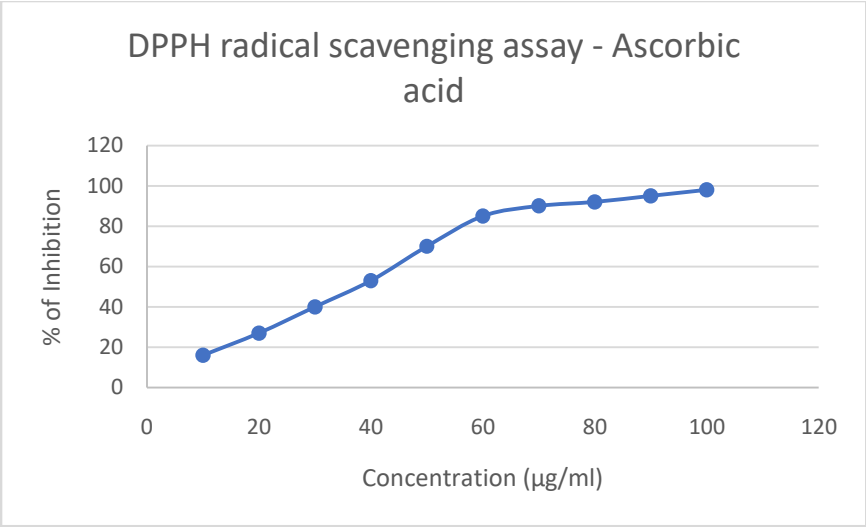

Figure 1: DPPH radical scavenging assayof Ascorbic acid

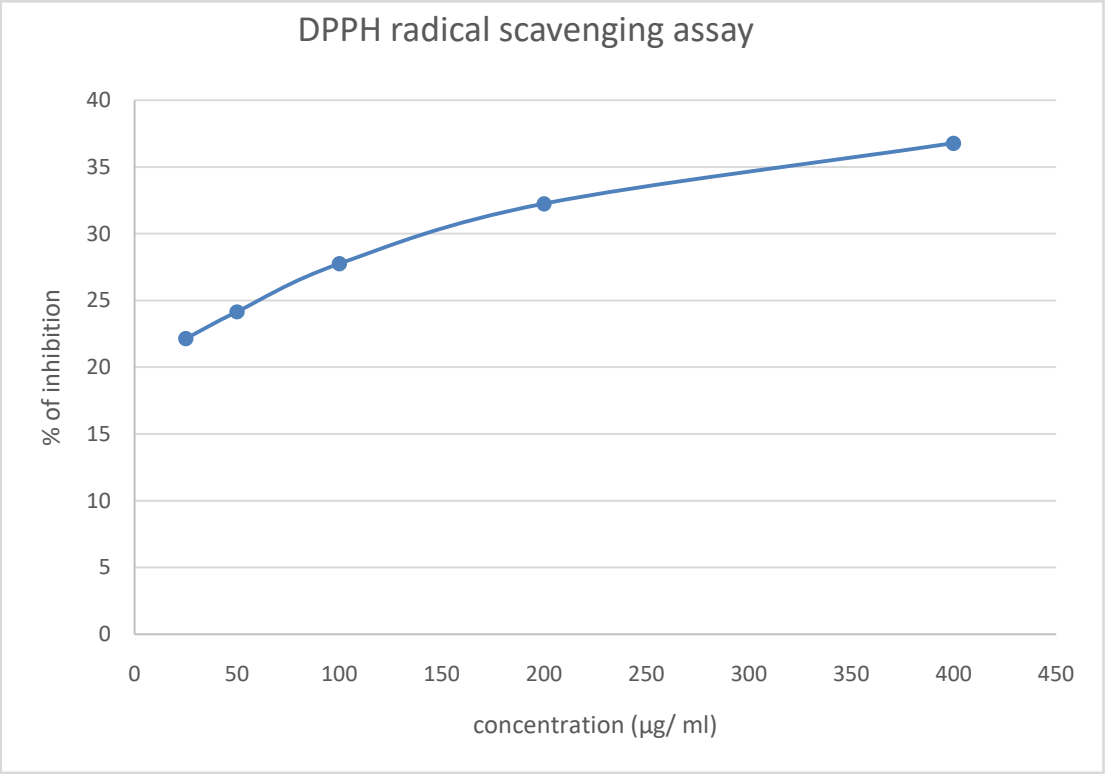

Figure 2: DPPH radical scavenging assayof the methanol extract

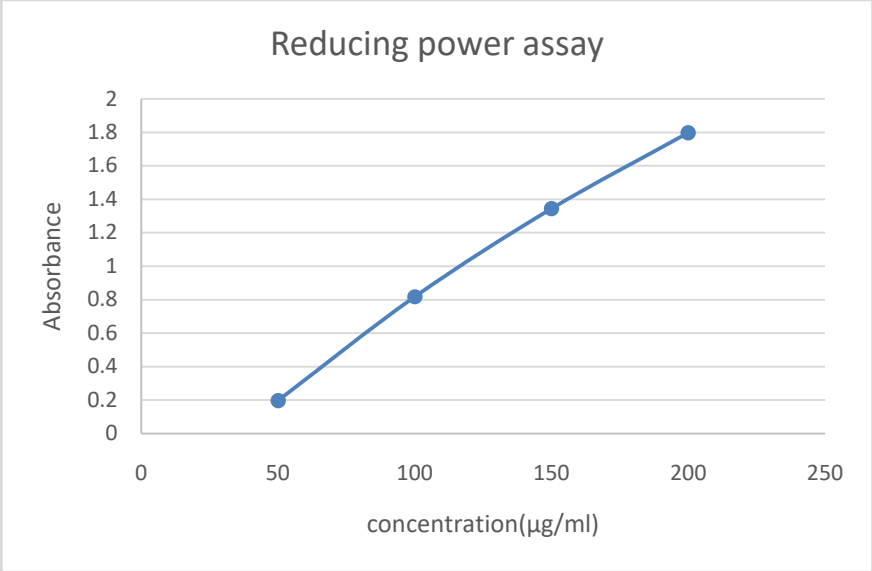

Figure3: Reducing power assay of the methanol extract

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 cholerae 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 12.33 ± 0.5 mm against Bacillus subtilisand 8.66 ± 0.5mm against Vibrio cholerae. The results indicated that the methanolic extract of A.triplinervishas significant antibacterial activity against Bacillus subtilis than Vibrio cholerae. The figure 4 illustrates the antibacterial activity of the extract against Bacillus subtilis, while figure 5 shows the antibacterial activity against Vibrio cholerae.

Figure 4: Antibacterial assay on B. subtilisFigure 5: Antibacterial assay on V. cholerae

Discussion:-

In the present study the methanol extract of A.triplinerviswas 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, flavonoids, reducing sugar, proteins, carbohydrates and alkaloids (Mamatha et al., 2017). The total phenol and flavonoids content in A.triplinerviswere also analyzed. Then it wasfound that the total phenol content was 56.6 ± 0.54mg gallic acid equivalent/g and total flavonoid content was7.43 ± 0.33mg catechin equivalent/g. The result indicates that fresh

leaves of A.triplinervishad 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. triplinerviswas 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.triplinervisexhibited the antioxidantactivity at varying concentrations of the leaf extracts. Free radical scavengingactivity increases with increase in concentration of the extract. In the present study, the fresh leafsample of A.triplinervisshowed 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 choleraewas 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 12.33 ± 0.5 mm against Bacillus subtilisand 8.66 ± 0.5 mm against Vibrio cholerae, indicating comparatively higher susceptibility of Bacillus subtilis to the plant extract.The observed antibacterial activity may be attributed by 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.triplinervisisa medicinally valuable plant in India and has been used from ancient times in traditional systems of medicine.This plant possesses 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 were estimated. The result indicates that fresh leaves ofA. triplinervishad high level of flavonoid and phenol contents.DPPHradical scavenging assay and reducing power assay proved that the plant has antioxidant activity.The antioxidant activity increases with increasing concentrations of the extract. The plant has antibacterial activity against Bacillus subtilisand Vibrio cholerae.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, and invivo validation are required to confirm its therapeutic potential of the plant.

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