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Original Article

Phytochemical Screening, HPTLC Fingerprinting and Invitro Antioxidant Activity of Root Extract of *Asparagus racemosus*

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ABSTRACT

Background: *Asparagus racemosus* is a climber shrub used in Indian medicine for centuries. It has been used as galactogogue and nerve tonic in folk medicine. The recent research on A. *racemosus* has revealed its disease fighting properties such as anti-bacterial, immunomodulatory, cardio protective, anti-stress, etc. Phytochemicals present in the plants are associated with their therapeutic capabilities. Hence, phytochemical screening of a therapeutic plant is essential. **Materials and Methods:** A preliminary qualitative screening of phytoconstituents present in the ethanol and aqueous extract of the plant was done. high-performance thin layer chromatography (HPTLC) was used to create a phytochemical fingerprint of the plant extract. Further, a series of antioxidant assays, i.e., 2,2-diphenyl-1-picrylhydrazyl (DDPH) radical, Nitric oxide (NO) radical, Superoxide (SO) radical and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assays were done. **Results:** The phytochemical screening and the HPTLC fingerprint showed the presence of phenolic compounds, flavonoids, glycosides, triterpenoids, saponins etc. They also showed free radical scavenging property and hence can be used as potential primary antioxidant. **Conclusion:** A preliminary screening created a phytochemical profile of *A. racemosus* extracts. These phytoconstituents may be linked to the various known therapeutic applications of the plant. This may aid in further extensive studies for identifying and isolating compounds with potential therapeutic value in *A. racemosus*.

Key Words: Antioxidants, Phytochemistry, Asparagus racemosus, chromatography, phytoconstituents, therapeutics.

INTRODUCTION

Plant based medicine system has been prevalent worldwide since time immemorial. According to WHO, about 80% of people rely on traditional and herbal medicine system for their primary health care[1]. The medicinal properties of plants can be attributed to the phytochemicals present in them. Phytochemicals are secondary metabolites that serve specific biological functions in the plant host and have potential disease-inhibiting abilities in animals[2]. Indian medicine systems like Ayurveda, Siddha and Unani use traditional herbs and plants against various diseases. Recently, there is a thrust in the research and development of traditional plant based drugs in modern medicine due to their remarkable versatility and proven efficacy[3]. It is estimated that 25% of drugs are plant based, suggesting the significant role of plants as a medicine source[4,5].

Asparagus racemosus Willd. is a perennial shrub belonging to the family Asparagaceae (previously subfamily Asparagae in the family Liliaceae) (Figure 1). It is commonly known as Shatavari (Hindi), Shimaishadavari (Tamil) or Satmuli (Bengali) and is found in various parts of India. *A. racemosus* is recommended in Ayurveda as galactogogue, aphrodisiac, nerve tonic, demulcent, diuretic, refrigerant and antispasmodic[6-8]. Further, the roots of this plant have been used to treat diseases related to spleen, liver, and other internal organs as well as for miscarriage threats[9].

Figure 1: *Asparagus racemosus* plant (Shatavari).

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The ethnopharmacological research in the last decade on *A. racemosus* revealed their immunomodulatory potential[10], phytoestrogenic effects[11], anti-diarrheal action[6], anti-dyspepsia action[12], anti-depressant action[13], anti-tussive action[14], immunoadjuvant[15], anti-bacterial action[16], anti-ulcerogenic action[7,17], anti-stress action[18], hypolipidemic potential[19] and cardio-protective ability[20]. Further, the extract of fresh roots of *A. racemosus* has been reported to have anti-neoplastic potential in female rats treated with 2,4-Dimethoxybenzaldehyde (DBMA)[21]. The disease-combating abilities of plants have been linked to their antioxidant potential[22]. Kamat *et. al.* reported the possible antioxidant activity of extracts of *A. racemosus* against oxidative damage caused by γ-radiation in rat liver mitochondria[23]. In addition, an antioxidant compound called Racemofuran was isolated and identified from the roots of *A. racemosus,* which showed free radical scavenging activity against DPPH[9].

The major constituents of *A. racemosus* are steroidal saponins, Shatavarins I-IV. Other phytoconstituents like Asparagamine, Racemosol, Sarsasapogenin and Kaempferol have also been isolated from various parts of *A. racemosus*[24-26]. The present study was performed to do a complete phytochemical screening of the aqueous and ethanol extract of *A. racemosus* plant. A complete study of the phytoconstituents in the plant will be helpful to evaluate the various nutritional and therapeutic compounds present in the plant. Here, we have employed the HPLTC technique to qualitatively and quantitatively assess the presence of various phytochemicals present in *A. racemosus* and their antioxidative ability was measured using various assays.

MATERIALS AND METHODS

Collection of plant material

The authenticated sample of dried aerial part of *A. racemosus* was procured from the Herbal Care and Cure Centre (Chennai, India). The collected sample was identified with the help of a botanist at the Central Drug Research Institute, Chennai, India.

Sample extraction

The root samples of *A. racemosus* were shade dried at 37ºC and powdered using pestle and mortar. For cold maceration method, 10gm of the root powder was placed in 100 mL of distilled water in a conical flask, plugged with cotton and then subjected to continuous stirring on a rotary shaker at 180-200 rpm for 72h. After 72h, the extract was filtered using a muslin cloth. Then the filtrate was lyophilized at -55°C. The lyophilized products were scraped and stored at 4°C in air-tight vials.

Phytochemical screening

Preliminary phytochemical screening of the cold aqueous and ethanolic extract was done to determine the presence of alkaloids, flavonoids, phytosterols, tannins/phenolic compounds, glycosides, carbohydrates, proteins and fats. For alkaloids, the extracts were treated with few drops of dilute HCl and filtered. The filtrates were then subjected to Dragendroff's reagent, Hager's reagent and Wagner's reagent. To qualitatively analyse the presence of phytosterol, 1gm of the extract was dissolved in few drops of dry acetic acid, 3ml of acetic anhydride was added followed by few drops of concentrated sulphuric acid.

For tannins and phenolics, three experiments with dilute ferric chloride solution (5%), 1% solution of gelatin with 10% NaCl, and 10% lead acetate solution were conducted separately. For flavonoids, Shinoda's test was performed. The extracts were dissolved in alcohol, to that a piece of magnesium followed by concentrated hydrochloric acid was added drop wise and heated to get magenta colour.

For carbohydrates, the extracts were dissolved in 5ml of distilled water and filtered and the filtrate was used to perform Molisch's, Fehling's and Barfoed's tests. The extracts were diluted in water and used for Biuret and Millon's test to detect proteins. To get the presence of fixed oils, the extracts were separately pressed between two filter papers. The oil stains on the paper indicate the presence of fixed oil. Further, saponification test by adding 0.5N alcoholic potassium hydroxide and phenolphthalein and heating the mixture in a water bath was performed to check the formation of soaps and thus the presence of fixed oils and fats.

DPPH free radical scavenging assay

The antioxidant activity of the aqueous extract of *A. racemosus* was estimated based on the ability to scavenge free DPPH radicals. Various concentrations of the extract were added to a methanolic 0.4 mM DPPH solution (0.1 ml) in a 96-well plate. The reaction mixture was shaken vigorously and allowed to stand for 30 min at 37°C. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract. The absorbance of the mixture was observed at 517 nm using UV-Vis microplate reader while ascorbic acid was served as a positive control**.**

The scavenging activity against DPPH was calculated using the following equation:

Scavenging activity (%) = [1 - (A1 - A2) / A0] x 100%

Where, A0 was the absorbance of the control (DPPH solution without the extract), A1 was the absorbance of DPPH solution in the presence of the extract and A2 was the absorbance without DPPH solution.

Superoxide radical scavenging activity

This assay is based on the capacity of the plant extract to inhibit nitro blue tetrazolium (NBT) upto 50% in the presence of the riboflavin-light-NBT system. The reaction medium contains 50 mM phosphate buffer (pH 7.6), 20µg riboflavin, and 12 mM EDTA. Different concentrations of the aqueous extract (5-100µg/ml), NBT 0.1 mg/3 ml and butylated hydroxytoluene (BHT) were taken in different test tubes and the same reagents were added. The reaction was started by illuminating the sample cuvette at regular intervals of 30 seconds and the increase in absorbance was measured at 590 nm upto 2.5 min. The superoxide radical scavenging activity was calculated using the formula:

% Inhibition of Superoxide radical = OD (extract absent) - OD (extract present) / OD (extract absent)

Nitric oxide radical scavenging assay

Sodium nitroprusside (SNP) in aqueous solution at a physiological pH, spontaneously generates NO which interacts with oxygen to produce nitrite ion, which is estimated using Griess reagent. The reaction mixture containing 2 ml of the *A. racemosus* aqueous extract at different concentrations and 50 mM SNP (0.5 ml) in 10 mM PBS was incubated at 37°C for 60 min. An aliquot (0.5 ml) of the incubation solution was pipetted out and diluted with 0.5 ml of Griess reagent (1% sulfanilamide in 5% H3PO4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED). The absorbance was immediately recorded at 540 nm. The absorbance from various concentrations of sodium nitrite salt treated the same way with Griess reagent was plotted for a standard curve. The capability to scavenge NO radicals was calculated using the following equation:

NO Scavenging activity (%) = [1 - (A1 - A2) / A0] x 100%

Where A0 was the absorbance of the control (the reaction mixture without the extract), A1 was the absorbance in the presence of the extract and A2 was the absorbance without Griess reagent. α-Tocopherol was used as a standard.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

ABTS radical cation was produced by the reaction of a 7 mmol/L ABTS solution with 2.45mmol/L potassium persulphate. The mixture was stored in the dark at room temperature for 12 hrs before use. The ABTS+ solution was diluted with ethanol and the absorbance has been measured at 734 nm. After addition of 25 µl of different concentrations of aqueous extract or standard to 2 mL of diluted ABTS+ solution, absorbance at 734 nm was read after 6 min. A standard curve was prepared by measuring the reduction in absorbance of ABTS+ solution at different concentrations of the extract. Appropriate blank measurements were carried out and the values recorded. Ascorbic acid was used as the positive control.

HPLTC fingerprinting

Densitometric HPTLC analysis of the *A. racemosus* plant extract was performed for the development of characteristic fingerprint for alkaloid, flavonoid, glycoside, phenolic and steroid profiles. Aqueous extract of the drug was centrifuged at 3000 rpm for 5 min. The supernatant was used as the test solution for HPTLC analysis. 2µl of the test solution and 2 µl of standard solution were loaded as 5 mm band length in the 3 x 10 Silica gel 60F 254 TLC plate by using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample-loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapor) with respective mobile phase and the plate was developed in the respective mobile phase up to 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The developed plate was sprayed with respective spray reagent and dried at 100°C in a hot air oven. The plate was photo-documented in UV 254 nm. The peak table and peak densitogram for each profile were noted. The software used was winCATS 1.3.4 version.

Statistical Analysis

The amount of effective concentration of the extract needed to inhibit free radicals by 50%, Inhibitory Concentration (IC50), was estimated from regression analysis between scavenging activities (%) versus various concentration of the extract. All antioxidant assays were performed in triplicates and the relative amount of Reactive Oxygen Species (ROS) (%) is shown as mean + SD.

RESULTS

Phytochemical screening

The preliminary qualitative phytochemical screening of the ethanol extract of *A.racemosus* revealed the presence of flavonoids, phytosterols, tannins/phenolic compounds, carbohydrates and protein in the ethanol extract and absence of alkaloids, glycosides and fixed oils and fats. The cold aqueous extract showed traces of alkaloid only during Wagner's test. In addition, it revealed the presence of flavonoids, tannins/phenolic substances, carbohydrates, proteins and fixed oils and fats. The complete results of this phytochemical screening are presented in Table 1.

HPTLC fingerprinting

The HPLTC method was used for fingerprinting of various phytoconstituents of *A. racemosus*. The analysis showed total six peaks with respective Rf values 0.18, 0.24, 0.36, 0.48, 0.65, and 0.72 for flavonoids, triterpenoids, saponins, glycosides, sterols and phenolic substances, as shown in Table 2, Figure 2 and Figure 3 show the HPTLC chromatograms.

The concentration of phenolic substances is found to be the highest through the densitometric analysis of the different peaks as shown in Table 2. The antioxidant and free radical scavenging activity of plant extracts are often associated with their phytoconstituents, especially the presence of phenolic compounds. The high presence of phenolic compounds, flavonoids and saponins indicate a high potential antioxidant activity of the *A. racemosus* extract.

Table 1. Qualitative Phytochemical screening of *A. racemosus* extracts.

Phytochemicals	Ethanolic Extract	Cold aqueous extract
Alkaloid		
Dragendroff's reagent	-	-
Hagers reagent	-	-
Wagner's reagent	-	+
Flavanoids		
Shinoda test	+	+
Saponins	+	+
Foam test	+	+
Phytosterols		
Salkowski test	+	-
Libermannbuchard test	+	-
Proteins		
Millon's test	+	+
Biuret test	+	+
Tannins/Phenolic substances		
Gelatin test	+	-
Lead Acetate test	+	+
Ferric chloride test	+	+
Glycosides		
Alkali test	+	+
Carbohydrates		
Molisch test	+	+
Fehling's test	+	+
Barfoed's test	+	+
Fixed oil & Fats		
Soap test	-	+
Saponification test	-	+

'+' indicates presence ;'-' indicates absence

Table 2. Peak table with Rf max values and calculated Peak area of different phytoconstituents present in aqueous extract of *A. racemosus*

Peak No	Rf value	Peak area	Assigned substances
1	0.18	861.3	Flavonoids
2	0.24	489.5	Triterpenoids
3	0.36	900.4	Saponins
4	0.48	2565.3	Glycosides
5	0.65	873.4	Sterols
7	0.72	4186.2	Phenolic substances

Antioxidant activity

DPPH molecule on accepting a hydrogen atom from the antioxidant reduces to DPPH2, changing the colour from purple to yellow. This change in absorbance is measured spectrophotometrically and the concentration of antioxidant is determined. The DPPH radical scavenging assay in this study revealed the IC_{50} of the aqueous *A. racemosus* extract to be 97.12 µg/ml. As shown in Table 3, Ascorbic acid, a well-known antioxidant was used as a standard to measure the activity of the extract.

The superoxide scavenging activity revealed the free radical scavenging activity of the *A. racemosus* is comparable to that of the standard scavenger, BHT. 100 µg/ml of both the standard and the test extract scavenged approximately 87.25% and 89.17% of NBT, respectively. As

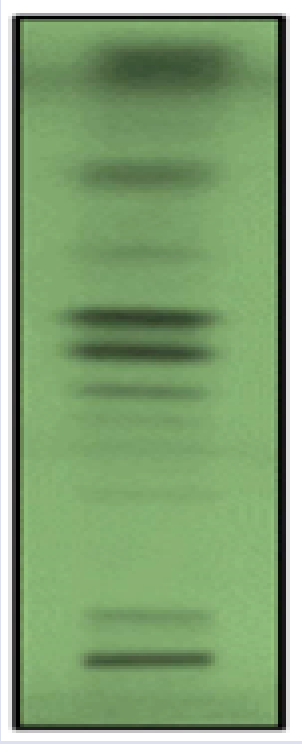

Figure 2: HPTLC chromatogram image of *A. racemosus* aqueous extract at 254nm.

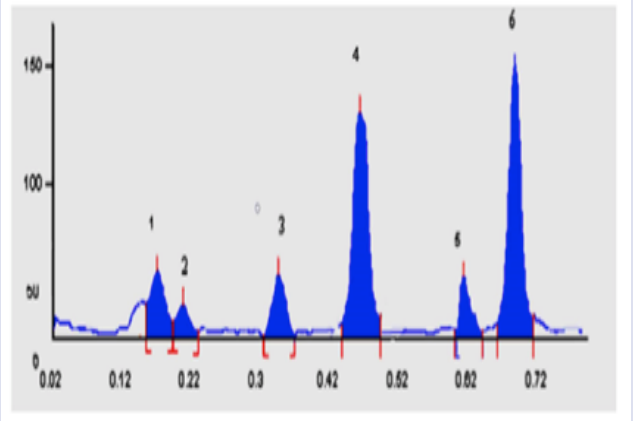

Figure 3: HPTLC chromatogram of aqueous extract of *A. racemosus* at 254nm showing different peaks (bands) of phytoconstituents.

shown in Table 4, the IC_{50} value of the extract was determined to be 21.97 µg/ml.

The Nitric oxide scavenging assay is based on the principle that the nitric oxide radical generated from the sodium nitroprusside can be measured by Greiss reduction. Tocopherol used as a standard. The high IC_{50} value of the extract, i.e., 500 µg/ml showed that the scavenging activity of the extract when compared to the standard was less effective (Table 5).

The results of the ABTS radical scavenging assay showed that the extract has an antioxidant effect in relatively higher concentrations compared to the standard. As shown in Table 6, 100 µg/ml of the standard Tocopherol when subjected to ABTS scavenging assay showed a similar percentage of inhibition to that of 1000 µg/ml of the *A. racemosus* extract.

DISCUSSION

Medicinal plants have been used in Indian traditional medicine and it has played crucial roles in the treatment of various diseases all over the world[27]. Phytotherapy in terms of herbal medicine is gaining traction worldwide as it is relatively safe compared to modern synthetic drugs. These disease-combating abilities of plants have been often linked to the bioactive compounds present in them. A preliminary screening of these plants for their phytochemical composition helps in creating a profile, which can be useful in the identification of compounds with therapeutic value. For example, Gupta et. al. screened *Curcumin longa* and *C. amada* extracts using HPTLC and identified three pharmacologically relevant curcuminoids, diaryl heptanoid curcumin, demethoxy curcumin, and bis-demethoxy curcumin[28]. *A. racemosus* has been employed in traditional medicine for various medical uses.

Table 3. DPPH scavenging activity of the *A. racemosus* aqueous extract and the standard Ascorbic acid and their IC_{50} values.

Sample	Conc µg/ml	% inhibition	IC_{50} µg/ml
Extract	10	17.23 ± 1.4	
	50	39.56 ± 2.8	
	100	51.48 ± 3.22	**97.12**
	250	65.47 ± 3.17	
	500	79.52 ± 5.4	
	1000	85.19 ± 7.3	
Ascorbic acid	100	86.17 ± 7.9	

Table 4. Superoxide radical scavenging activity of the *A. racemosus* aqueous extract and the standard BHT and their IC_{50} values.

Sample	Conc µg/ml	% inhibition	IC_{50} µg/ml
Extract	5	17.23 ± 1.4	
	10	39.56 ± 2.8	
	25	51.48 ± 3.22	**21.97**
	50	65.47 ± 3.17	
	100	79.52 ± 5.4	
BHT	100	86.17 ± 7.9	

Table 5. Nitric oxide radical scavenging activity of the *A. racemosus* aqueous extract and the standard Tocopherol and their IC_{50} values.

Sample	Conc µg/ml	% inhibition	IC_{50} µg/ml
Extract	10	16.15 ± 0.97	
	50	22.18 ± 1.8	
	100	34.19 ± 2.4	**500**
	250	41.18 ± 3.5	
	500	50.17 ± 4.1	
	1000	58.55 ± 4.9	
Tocopherol	100	59.71 ± 0.52	

Table 6. ABTS radical scavenging activity of the *A. racemosus* aqueous extract and the standard Ascorbic acid and their IC_{50} values.

Sample	Conc µg/ml	% inhibition	IC_{50} µg/ml
Extract	10	12.86 ± 0.26	
	50	18.29 ± 1.05	
	100	29.54 ± 1.8	**592.67**
	250	30.14 ± 0.69	
	500	42.18 ± 0.34	
	1000	57.46 ± 0.21	
Tocopherol	100	58.74 ± 0.38	

Recent ethnopharmacological research has added scientific data to the existing claims from folk medicine. The major constituents reported to be in *A. racemosus* are saponins called Shatavarins[26]. In order to understand the potential therapeutic properties, a complete screening of the extract is needed to analyze the different phytochemicals present in it. Thus, a preliminary phytochemical screening of the *A. racemosus* extract was performed in order to create a profile of their phytoconstituents.

HPTLC is an important semi-quantitative and quantitative tool for the phytochemical analysis of herbal drugs and formulations. The major advantage being a parallel screening of several samples can be done[29]. For example, phytochemical analysis of *Strychnos nux-vomica* extract through HPTLC helped in analyzing their phytoconstituents and identification of medically relevant compounds[30]. In addition, HPTLC can be used as a diagnostic tool for the identification of plant and as a phytochemical marker like in case of *Pisonea aculeata*[31]. In our study, the presence of phenolic acids, triterpenoids, alkaloids, flavonoids, saponins, etc. revealed that they are responsible for the medicinal and therapeutic properties of the plant. The analysis helped in creating a phytochemical fingerprint of *A. racemosus*. The pharmacological studies conducted on the plant indicated their potential in the treatment of conditions such as menopausal symptoms, neurodegenerative disorders, diarrhoea, dyspepsia, etc.[26,32]. The HPTLC results may point a finger at the presence of these polyphenols as responsible for the known therapeutic potential of the plant.

There is increasing evidence of chronic disorders due to the production of free radicals or ROS. The high reactivity of these free radicals leads to oxidative stress damaging the cells and ultimately leading to life threatening disorders. Oxidative stress is involved in various acute and chronic disorders like cardiovascular, kidney, neurodegenerative, cancer, etc.[33]. Phytoconstituents of plants are associated with their free radical scavenging properties. Due to the presence of specific compounds responsible for their antioxidant properties, drugs from plant sources have been on a market rise. The study on antioxidant properties of more plants helps in the identification of compounds that may be discovered for combating various diseases[34]. The antioxidant assays revealed the ability of the *A. racemosus* extract to scavenge free radicals. This indicates that *A. racemosus* can be studied further for their strong antioxidant compounds. The DPPH assay showed high free radical scavenging and this may be due to the presence of Racemofuran, a benzofuran which has been reported to have DPPH free radical scavenging activity[9].

Due to the crucial role antioxidants play in disease management, drug discovery should be more focused towards plant sources, which are known to have immense antioxidant potential. Creating the phytochemical profile of these plant sources may help in the quick identification and isolation of medically relevant constituents aiding in the faster and efficient discovery of this safer option.

CONCLUSION

A preliminary phytochemical screening of *A. racemosus* plant extract was necessary and performed to understand the bioactive profile of the plant. This revealed a presence of polyphenols including phenolic acids, flavonoids, saponins, glycosides, etc. The HPTLC fingerprinting is an interesting tool to further quantitatively assess the phytoconstituents present in the extract. The high presence of phenolics can be linked to their high free radical scavenging activity. These experiments help in creating a scientific profile for a traditionally well-known plant. These data along with the plethora of opportunities that modern medicine provides may be useful in identifying a greater number of natural molecules and curating medicines that serve all sections of the society with no harmful effects.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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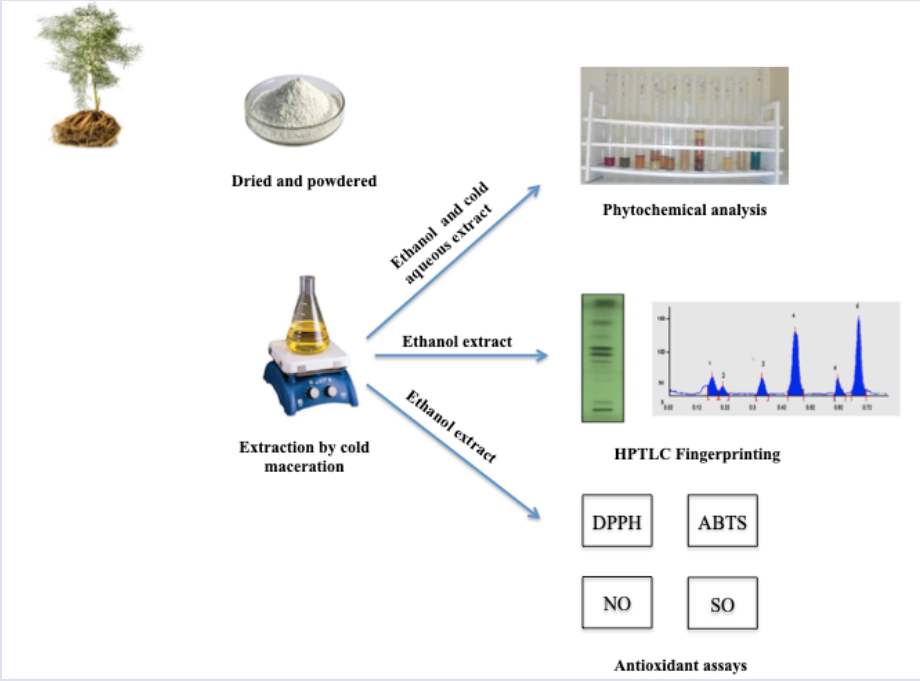
GRAPHICAL ABSTRACT



SUMMARY

- *A. racemosus* plant extract was prepared using ethanol and water.
- The plant extracts were subjected to phytochemical screening that revealed the presence of various phytoconstituents like phenolics, flavonoids, glycosides, proteins, carbohydrates etc.
- A further HPTLC fingerprinting created a profile of the bioactive compounds present in the ethanol extract. It showed the highest presence of phenolics and then glycosides. Peaks corresponding to saponins, flavonoids sterols, triterpenoids were also observed.
- The free radical scavenging activity was assessed and the DPPH, ABTS, Nitric Oxide, Superoxide assays showed the antioxidant potential of the extract.
- These assays help in the identification of molecules that may be responsible for the therapeutic potential of the plant.

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