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IN-VITRO ASSESSMENT OF ANTIOXIDANT AND ANTIPROLIFERATIVE EFFICACY OF *ANDROGRAPHIS PANICULATA*

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Keywords:

Andrographis paniculata, Antioxidant activity, DPPH, Reducing power, Antiproliferative, MTT

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ABSTRACT: The present study investigated the antioxidant and antiproliferative efficacy of *Andrographis paniculata*, a well-known medicinal plant, using *in-vitro* systems. The dried leaves of *A. paniculata* were powdered and subjected to successive solvent extraction taking from polar to non-polar solvents (descending polarity) and simultaneously from non-polar to polar solvents (ascending polarity). Solvents used for extraction were hexane, chloroform, ethyl acetate, acetone, methanol, and water. All the extracts were screened for the presence or absence of various secondary metabolites. The antioxidant activity of all extracts at 100 µg/ml was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Further DPPH assay and reducing power assay were used to check the free radical scavenging capacity of methanolic and water extracts at their lower concentrations. The methanolic and water extracts of *A. paniculata* exhibited maximum DPPH inhibition of 20.75% and 17.71% respectively, at 100 µg/ml. A positive correlation between reducing power and concentration was found. Thin Layer Chromatography (TLC) of methanolic and water extracts of *A. paniculata* was performed. The antiproliferative effect of different concentrations of methanolic and water extracts on C6 glioma cells was investigated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The methanolic extract exhibited 50% inhibition of proliferation of C6 glioma cells at much lower concentration (12.5 µg/ml) than did the water extract (200 µg/ml). This study is a novel approach towards the antioxidant and antiproliferative role of methanolic and water extracts of *A. paniculata* which suggests its anticancer potential.

INTRODUCTION: Medicinal plants are being used in traditional system of medicine from hundreds of years in many countries of the world due to their therapeutic properties [1], which are very useful in healing various diseases and the advantage of these plant-based traditional medicines is their being 100% natural [2].

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Andrographis paniculata, highly reputed as "Kalmegh" and "King of Bitters", is one of the important herbs comprising of several medicinal properties. *A. paniculata* is well known traditional medicinal plant species with a bright economic horizon, belonging to *Acanthaceae* family, found throughout Southeast Asia [3].

The bitterness of *A. paniculata* is related to its various pharmacological properties [4]. Diterpenoids and flavonoids are the main chemical constituents of *A. paniculata*, which are believed to be responsible for most of the biological activities of this plant [5].

Free radicals, also known as reactive oxygen species (ROS), play a role in the etiology of several major diseases, including cancer. Reactive oxygen species (ROS) cause DNA strand breakage, sister chromatid exchanges and DNA–DNA and DNA–protein cross-links, in addition to base modifications, which have been associated with carcinogenesis [6]. Since free radicals play a key role in the pathology of diseases, the supply of antioxidants, *via* the food chain, is of great importance for a healthy life [7].

The present study aimed to elucidate whether leaf extracts of *A. paniculata* has antioxidant and antiproliferative activity. As many of the diseased conditions (like cancer, atherosclerosis, cardio-vascular diseases, and degenerative disorders) are due to oxidative stress, the antioxidant potential of leaf extracts of *A. paniculata* was studied by performing DPPH and reducing power assays.

This study was undertaken because the leaves of *A. paniculata* are well known for their antioxidant property [8]. C6 glioma cell line was used, and MTT assay was done to study the antiproliferative effect of methanolic and water leaf extracts of *A. paniculata*.

MATERIALS AND METHODS:

Materials: 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma Aldrich, USA. All other chemicals like ninhydrin reagent, ammonia, pyridine, sodium nitroprusside, ether, lead acetate, acetic acid, sodium hydroxide, potassium hydroxide, dilute hydrochloric acid, dilute acetic acid, concentrated sulphuric acid, ferric chloride, acetic anhydride, L-ascorbic acid, sodium hydroxide, trichloroacetic acid, phosphate buffer, potassium ferricyanide, ferric chloride, and other solvents were of analytical grade from CDH.

DMEM (Dulbecco's Modified Eagle's Medium), Hank's balanced salt solution (HBSS) and FBS (foetal bovine serum) from HIMEDIA, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Life Technologies.

Methods:

Preparation of Different Extracts of *A. paniculata*: Extraction method involved separation of medicinally active fractions of plant tissue from inactive/inert components by using selective solvents and extraction technology. Solvents diffuse into the solid plant tissues and solubilize compounds of similar polarity [9].

The dried leaves of *A. paniculata* were powdered and subjected to successive solvent extraction taking from polar to non-polar solvents (descending polarity) and simultaneously from non-polar to polar solvents (ascending polarity). Solvents used were water, methanol, acetone, ethyl acetate, chloroform, and hexane.

For extraction according to ascending polarity, 25 gram of powered plant leaf material was dissolved in 100 ml of hexane and was placed in orbit shaker at 130 rpm for 24 h at room temperature. The extract was filtered with the help of muslin cloth, and the filtrate was then subjected to centrifugation at 12000 rpm for 30 min at 5 ºC.

The residue obtained was then dissolved in chloroform and was subjected to the same processing as shown in **Flow chart 1**. Similarly, extraction was performed in descending polarity from polar to non-polar solvents as described in **Flow chart 2**.

Estimation of the Concentration of Extracts: An empty Petri plate was weighed. 1ml of descending water extract was added to it. The Petri plate was kept in the incubator overnight at 37 ºC and again weighed. The concentration was calculated using the formula:

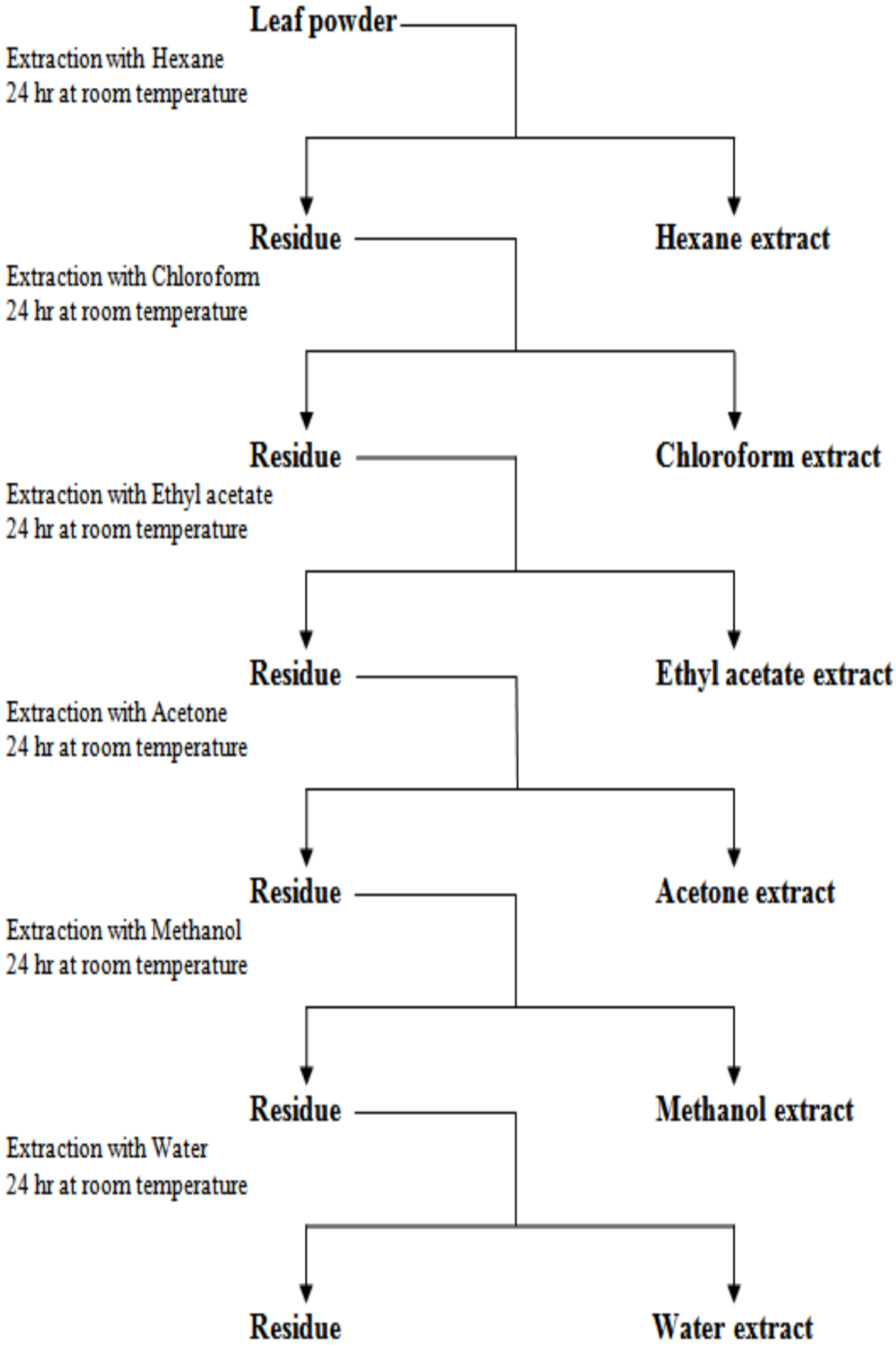
$$\text{Conc.} = y - x = a \text{ g/ ml}$$

Y = weight of Petri plate after incubation

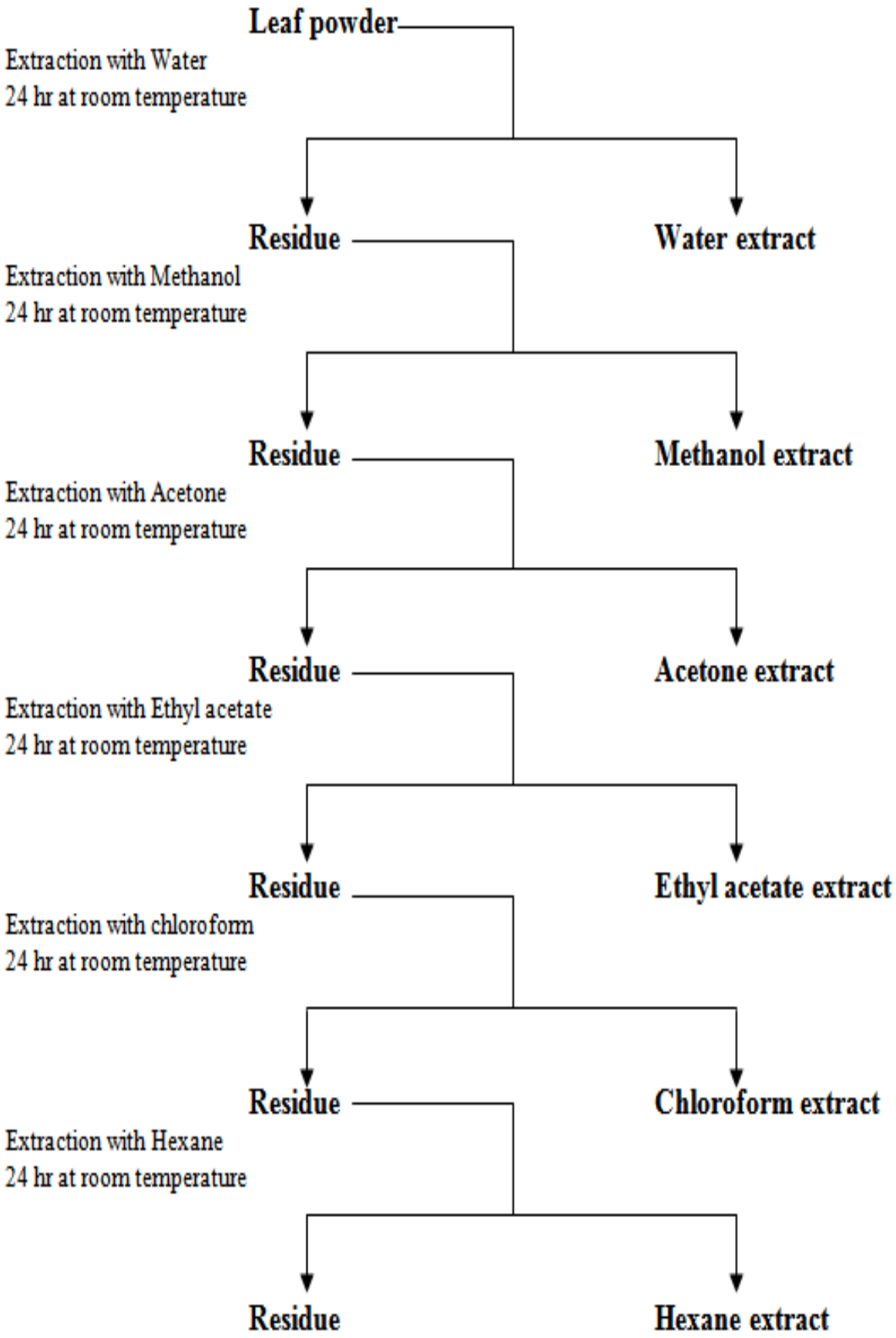
X = weight of empty Petri plate

The concentration of all extracts was calculated similarly.

Phytochemical Screening of Plant Extracts: Standard screening tests of extracts were carried out for various plant bioactive constituents. All the extracts were screened for the presence or absence of various secondary metabolites like amino acids, anthraquinones, glycosides, steroids, flavonoids, phytosterols, saponins, tannins, and triterpenoids.



FLOW CHART 1: EXTRACTION BY MACERATION OF LEAF POWDER OF *A. PANICULATA* BY INCREASING ORDER OF SOLVENT POLARITY



FLOW CHART 2: EXTRACTION BY MACERATION OF LEAF POWDER OF *A. PANICULATA* BY DECREASING ORDER OF SOLVENT POLARITY

Antioxidant Activity: Antioxidant activity of the extracts was analyzed by two methods:

DPPH Assay: The DPPH radical scavenging assay [10], an easy, rapid, and sensitive method was used to determine the antioxidant activity. The radical scavenging activities of the plant extracts were determined using UV spectrophotometer at 550 nm. DPPH (0.1 mM) was prepared in methanol. 2 ml of DPPH was added to 300 µl of 100 µg/ml concentration of each extract. Blanks were prepared to contain 2 ml of DPPH and 300 µl of solvent in which extract was prepared. Then the absorbance was measured at 550 nm at zero and twenty minutes. A decrease in the absorbance of the reaction mixture after twenty minutes indicates free radical scavenging activity. The further assay was performed on different concentrations of ascending polarity methanolic and descending polarity water extracts (chosen on the basis of the DPPH assay results and presence of maximum phytochemicals) starting from a concentration of 10 to 100 µg/ml. Vitamins C (Ascorbic acid) was used as the antioxidant standard. The radical scavenging activity was calculated as % inhibition of DPPH.

% Inhibition = A control – A test × 100 / A control

A control is the absorbance of the blank and
A test is the absorbance of the extract.

Reducing Power Assay: Antioxidant activity was also determined by Ferric ion Reducing Antioxidant Power (FRAP) assay [11]. Different concentrations (10 to 190 µg/ml) of the ascending polarity methanolic and descending polarity water extracts were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1% w/v). The mixture was incubated at 50 ºC for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to each mixture, which was then centrifuged at 1036 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (2.5 ml, 0.1%). The absorbance was measured at 700 nm. A blank was prepared without adding extract. Increased absorbance of the reaction mixture indicates an increase in reducing power.

Thin-layer Chromatography: Thin-layer chromatography (TLC) was employed in this study to analyze the compounds present in the ascending polarity methanolic and descending polarity water extracts. Normal phase silica gel pre-coated TLC plates were used. Thin Layer Chromatography (TLC) of methanolic and water extract of *A. paniculata* was performed on chloroform: methanol (191:9) and chloroform: methanol (7:3) solvent systems respectively. The distances of bands from origin were measured, and R_f (retention factor) values were calculated.

Antiproliferative Assay: Antiproliferative activity of ascending polarity methanolic and descending polarity water extract was tested on C6 glioma cells using MTT test [12]. Undifferentiated cells were subcultured by trypsinization (0.1% trypsin) when they reached 70-80% confluency. The experiment was performed in 96 well plates. Two replicates were used for the analysis of each concentration. C6 glioma cell line (40000 cells/ml) was treated with serially diluted concentrations of extracts for 24 h. The optic absorbance of each sample was measured at a wavelength of 595 nm using ELISA Reader.

% Viability = Mean Absorbance of Sample × 100 / Mean Absorbance of Control

The relationship between the concentration of extracts of *A. paniculata* and % viability was determined.

RESULTS AND DISCUSSION: Although *A. paniculata* has long served as traditional medicine, very few authentic studies evaluating its properties are available. *In-vitro* studies on *A. paniculata* have shown the presence of many bioactive constituents [13] having pharmacological as well as medicinal properties which include its activities as anti-snakebite [14, 15], anti-diabetic [16, 17, 18, 19, 20], anti-cancer [16, 21, 22, 23, 24, 25, 26, 27, 28], cytochrome inhibitory [29, 30], anti-viral [16, 31], anti-protozoal [32], anti-malarial [33], cardio-protective [34], anti-inflammatory [16, 35, 36, 37, 38, 39, 40], antioxidant [41, 42, 43, 44], cerebro-protective [45, 46], hepato-protective [47, 48], anti-bacterial [16, 42, 44, 49, 50] and anti-human immunodeficiency virus (HIV) [51].

The concentration of ascending polarity hexane, chloroform, ethyl acetate, acetone, methanol, and water extract was 6, 5, 3, 2, 3, and 7 mg/ml, respectively. The concentration of descending polarity water, methanol, acetone, ethyl acetate,

chloroform, and hexane extract was 18, 4, 6, 4, 3, and 6 mg/ml, respectively. The maximum concentration was that of descending polarity water extract (18 mg/ml).

The phytochemical screening of the plant extracts showed the presence of amino acids, flavonoids, glycosides, saponins, steroids, tannins, and triterpenoids as elaborated in **Table 1** and **2**. Most of the bioactive components were showed to be present in ascending polarity methanolic and descending polarity water extract. These results are consistent with the findings on aqueous extract of *A. paniculata,* which show the presence of glycosides, saponins, tannins, and alkaloids, but not of anthraquinones [52]. Diterpenoids and flavonoids are the main chemical constituents of *A. paniculata*, which are believed to be responsible for the most biological activities of this plant [5].

Phenolics, alkaloids, terpenoids, and cardiac glycosides detected in the extracts are compounds that have been documented to possess medicinal properties and health-promoting effects [53, 54, 55, 56].

TABLE 1: PHYTOCHEMICAL CONSTITUENTS OF ASCENDING POLARITY EXTRACTS OF *A. PANICULATA*

Bioactive Components	Extracts					
	Hexane	Chloroform	Ethyl acetate	Acetone	Methanol	Water
Amino acids	-	-	-	-	-	+
Anthraquinones	-	-	-	-	-	-
Flavonoids	-	-	-	-	+	+
Glycosides	-	-	-	+	-	-
Phytosterols	-	-	-	-	-	-
Saponins	-	-	-	-	+	-
Steroids	-	+	-	-	+	+
Tannins	-	-	-	-	+	+
Triterpenoids	-	-	-	-	-	+

(+) present, (-) absent

TABLE 2: PHYTOCHEMICAL CONSTITUENTS OF DESCENDING POLARITY EXTRACTS OF *A. PANICULATA*

Bioactive Components	Extracts					
	Water	Methanol	Acetone	Ethyl acetate	Chloroform	Hexane
Amino acids	+	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-
Flavonoids	+	-	-	-	-	-
Glycosides	-	-	+	-	-	-
Phytosterols	-	-	-	-	-	-
Saponins	+	-	-	-	-	-
Steroids	+	+	-	-	-	-
Tannins	+	-	-	-	-	-
Triterpenoids	+	-	-	-	-	-

(+) present, (-) absent

All extracts except ascending polarity hexane showed a decrease in absorbance, but significant decrease in absorbance was showed by ascending polarity methanolic and descending polarity water extract as depicted in **Fig. 1** and **2**. Further assays were performed on different concentration of water and methanol extract starting from 10 to 100 µg/ml. All concentrations significantly lowered the DPPH free radical scavenging activity. The ascending polarity methanolic extract of *A. paniculata* had maximum inhibition of 20.75% at 100 µg/ml. The descending polarity water extract of *A. paniculata* appeared to have maximum inhibition of 17.7% at 100 µg/ml, as shown in **Fig. 3** and **4**. These results are consistent with the research done on leaf, fruit, and stem extracts of *A. paniculata* [57]. However, DPPH scavenging assay is not capable of detecting all anti-oxidants, and therefore, there is a need to use several assays to demonstrate antioxidant activities.

The results of the reducing power assay on ascending polarity methanolic and descending polarity water extracts showed a linear increase in the intensity of blue color as the concentration of extracts increases as depicted in **Fig. 5**.

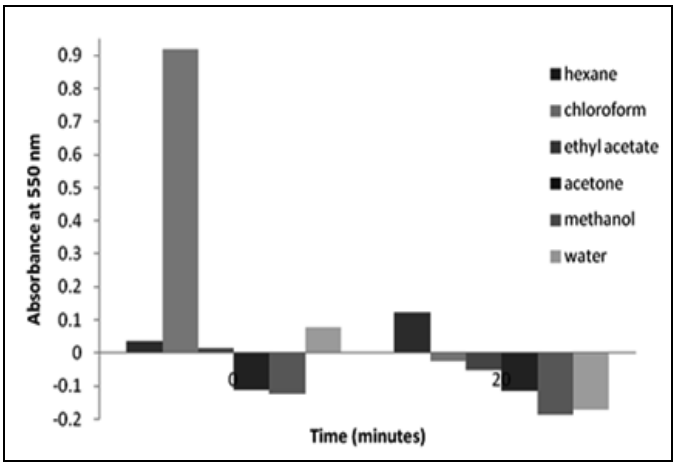


FIG. 1: INHIBITION OF DPPH BY ASCENDING POLARITY EXTRACTS

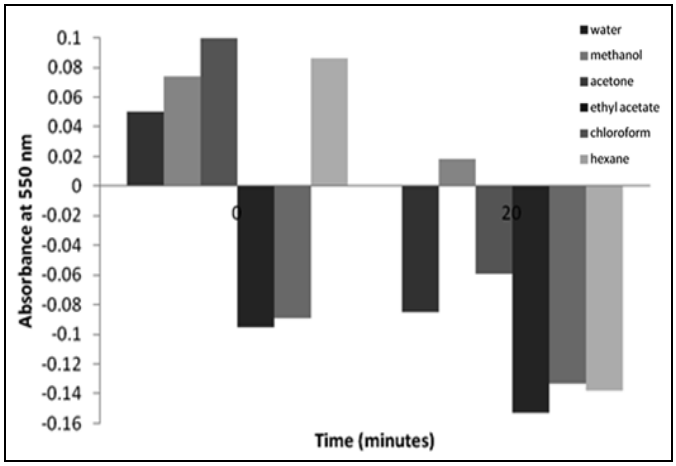


FIG. 2: INHIBITION OF DPPH BY DESCENDING POLARITY EXTRACTS

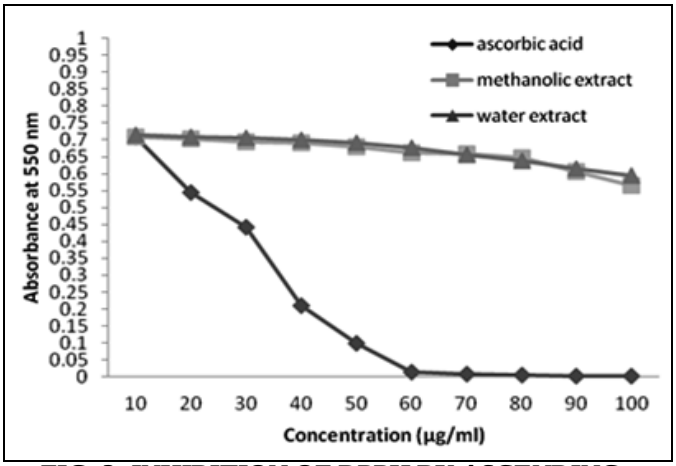


FIG. 3: INHIBITION OF DPPH BY ASCENDING POLARITY METHANOLIC AND DESCENDING POLARITY WATER EXTRACTS OF *A. PANICULATA*

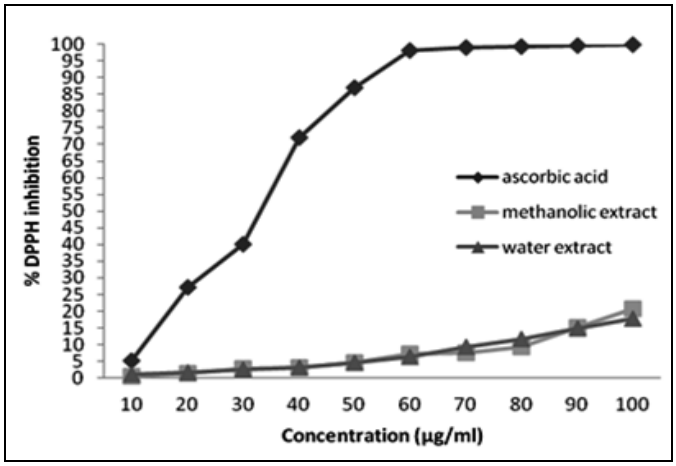


FIG. 4: INHIBITION OF DPPH BY ASCENDING POLARITY METHANOLIC AND DESCENDING POLARITY WATER EXTRACTS OF *A. PANICULATA*

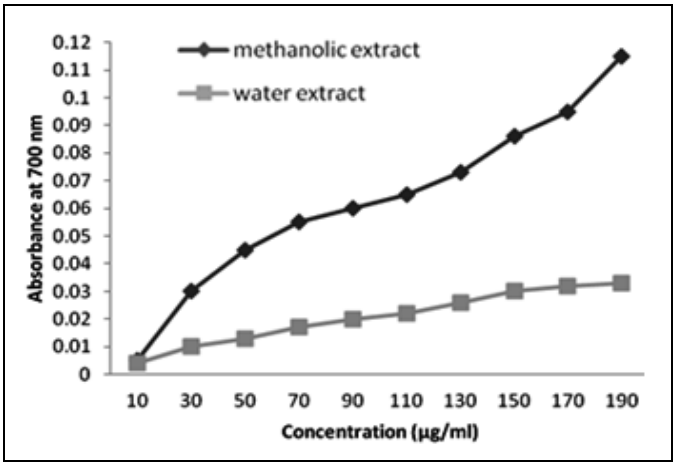


FIG. 5: REDUCING POWER ASSAY ON DIFFERENT CONCENTRATIONS OF ASCENDING POLARITY METHANOLIC AND DESCENDING POLARITY WATER EXTRACTS OF *A. PANICULATA*

An idea about the polarity of various chemical constituents is also obtained while performing TLC analysis. Compound showing high R_f value in the less polar solvent system has low polarity and with less R_f value have high polarity.

Six bands were obtained on staining the TLC plates with iodine vapors for ascending polarity methanolic extract. The distances of bands 1 to 6 from origin were 1.1, 2.1, 2.5, 3.5, 4 and 4.7 respectively.

The solvent front was 7.9 cm, as shown in **Fig. 6**. The R_f values of bands 1 to 6 were 0.13, 0.26, 0.31, 0.44, 0.50, 0.59 respectively. Five bands were obtained for descending polarity water extract. The distances of bands 1 to 5 from origin were 1.2, 2, 3.4, 4.3 and 5.1 respectively. The solvent front was 7.9 cm, as shown in **Fig. 7**. The R_f values of bands 1 to 5 were 0.15, 0.25, 0.43, 0.54, and 0.64 respectively.

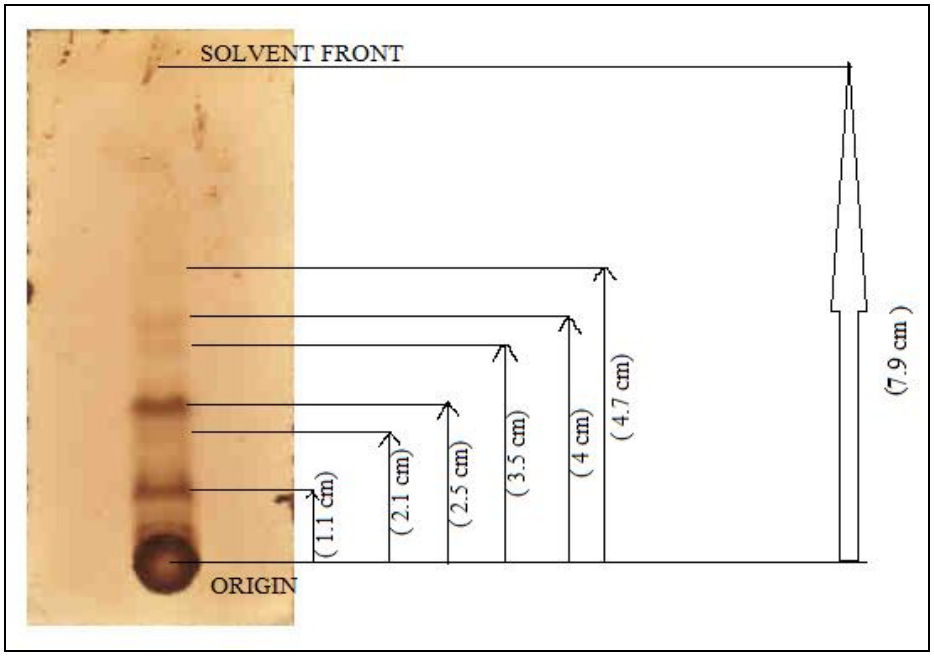


FIG. 6: THIN LAYER CHROMATOGRAPHY OF ASCENDING POLARITY METHANOLIC EXTRACT OF *A. PANICULATA*

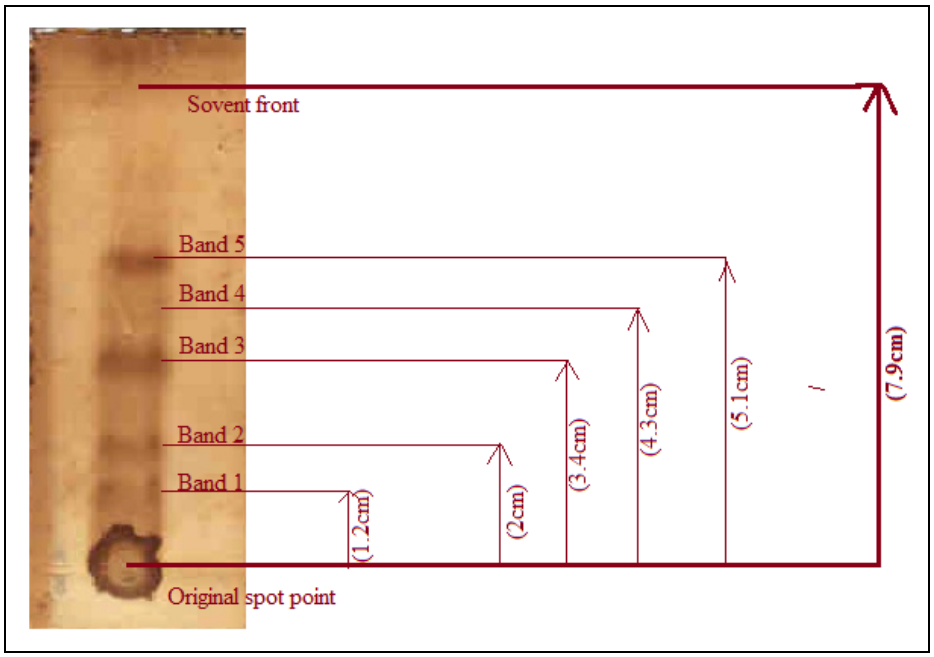


FIG. 7: THIN LAYER CHROMATOGRAPHY OF DESCENDING POLARITY WATER EXTRACT OF *A. PANICULATA*

The minimum viability (16.83%) was at the highest concentration of the ascending methanolic extract (200 µg/ml), and maximum viability (99.13%) was at the lowest concentration of extract (1.562 µg/ml). Similarly, the minimum viability (55.17%) was at the highest concentration of the descending polarity water extract (200 µg/ml) and maximum viability (98.11%) was at the lowest concentration of extract (1.562 µg/ml). The ascending polarity methanolic extract exhibited 50% inhibition of proliferation of C6 glioma cells at much lower concentration (12.5 µg/ml) than did the descending polarity water extract (200 µg/ml) as shown in **Fig. 8, 9** and **10**.

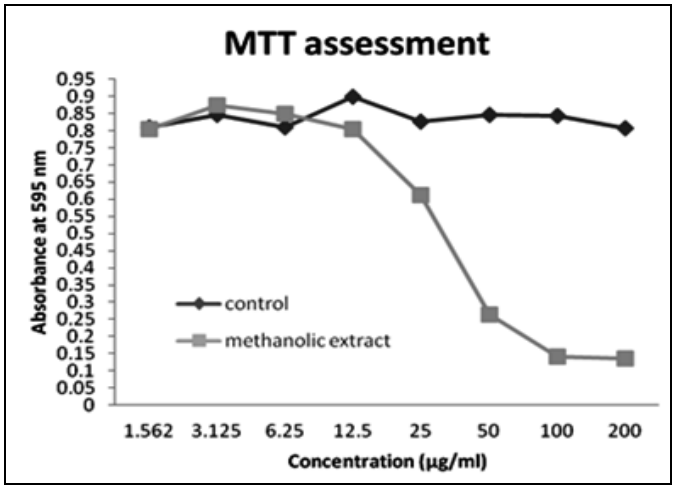


FIG. 8: EFFECT OF ASCENDING POLARITY METHANOLIC EXTRACT OF *A. PANICULATA* ON C6 GLIOMA CELL LINE ASSESSED BY MTT ASSAY

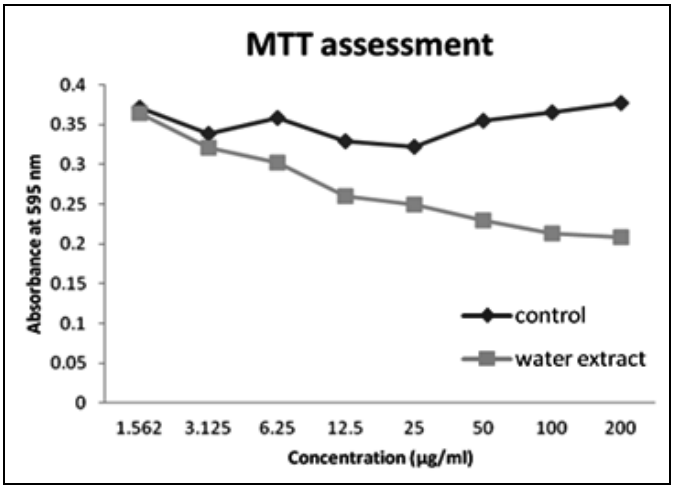


FIG. 9: EFFECT OF DESCENDING POLARITY WATER EXTRACTS OF *A. PANICULATA* ON C6 GLIOMA CELL LINE ASSESSED BY MTT ASSAY

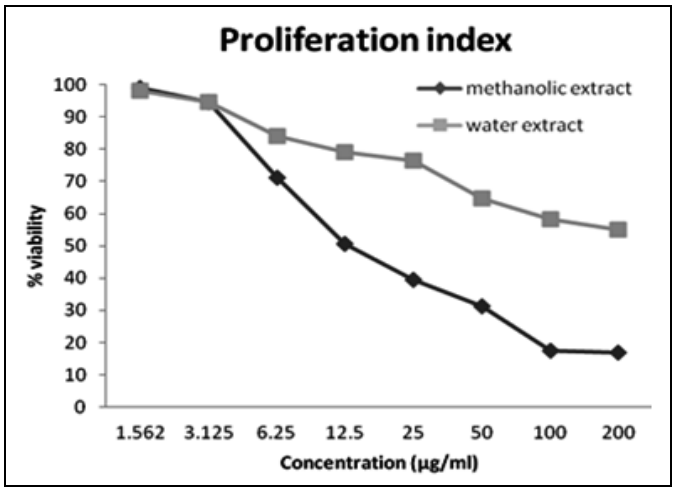


FIG. 10: EFFECT OF ASCENDING POLARITY METHANOLIC AND DESCENDING POLARITY WATER EXTRACTS OF *A. PANICULATA* ON PROLIFERATION OF C6 GLIOMA CELL LINE ASSESSED BY MTT ASSAY

CONCLUSION: Although *A. paniculata* is most commonly used in Indian Ayurvedic medicine, but the mechanistic aspects of its effects and isolation of active components are still not explored. The results of the present study provide the evidence of the antioxidant and antiproliferative effect of ascending polarity methanolic and descending polarity water leaf extracts of *A. paniculata.*

The presence of various bioactive constituents indicates the therapeutic potentials of *A. paniculata.* Thus this study is a novel approach towards assessing the cytoprotective and anticancerous role of leaf extracts of *A. paniculata.* It shows great potential as a safe and effective antineoplastic agent. Further characterization of the *A. paniculata* leaf extracts to identify the active antiproliferative components will be of immense therapeutical value.

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CONFLICT OF INTEREST: Nil

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