Research Article

Evaluation of Phytochemical and Anticancer Potential of *Aphanamixis polystachya (Wall.)* Parker Stem Bark using *in-vitro* Models

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ABSTRACT

Cancer is one of the leading causes of mortality worldwide. Many of the Meliaceae plants traditionally reported to possess antitumor activity. The present study was carried out to evaluate the anticancer activity for Methanolic extract and N-Hexane fraction of *Aphanamixis polystachya stem* bark. The present research work had carried out on laboratory level assay to avoid the use of different animal models. The antioxidant activities were examined by Three different methods namely nitric oxide scavenging activity, reducing power, Scavenging of hydrogen peroxide The dried stem bark *of Aphanmixis polystachya (wall.) Parker.* Were successively extracted with petroleum ether, chloroform and Methanol. N-Hexane fraction of Methanolic extracts are subjected to in vitro Antimitotic assay by Allium cepa root inhibition The successive Methanol extract and it's N-Hexane fraction was subjected to in vitro anticancer activity by SRB assay HeLa, MCF7 a cell lines. The antimitotic assay by onion root method was selected because this is easy to done and give fastest promising results. Onion was selected for the antimitotic assay which shows the root growth inhibition that compared with standard antimitotic drug (Vincristine). Methanolic extract and its N-Hexane fraction of Aphanamixis polystachya stem bark powder shows most promising anticancer activity and antioxidant activity.

Keywords: *Aphanamixis polystachya,* Antimitotic (Allium cepa root inhibition), Antioxidant, Anticancer by SRB assay.

INTRODUCTION

Cancer is one of the most fatal diseases in human population and one of the most frequent causes of death worldwide. To prevent the cancer, synthetic and natural sources are used in alone or combination. Today due to resistance of different allopathic medicines natural source is preferred mainly to block the development of cancer in human. Plant shows different chemical moiety including flavonoids, terpenoids, and steroids[1-3] which have the Pharmacological properties like antiulcer[i], anti hyperlipidemic[ii], antioxidant, cytotoxic as well. Ayurveda is conventional medicinal systems of Indian's. Now the whole world is interested in India's *Ayurveda* and other traditional medicine systems. The demand of medicinal plants is increasing day by day in both developing as well as developed countries as a result of recognition of the non-narcotic nature, lack of side effects and easily availability of many herbal drugs. Most often the medicinal plants are collected from the wild. Ayurveda approves *Aphanamixis polystachya* (*A. polystachya*) in liver and spleen disorders, tumors, ulcer, dyspepsia, intestinal worms, skin diseases, leprosy, diabetes, eye diseases, jaundice, hemorrhoids, burning sensation, arthritis and leucorrhoea .[6] The Santanol ethnic people of India employ the wood extract in healing cancerous wound.[6] The fruits are used as anthelmintic, laxative, refrigerant and is said to be useful against ulcers and rheumatism while the seed oil is used as a liniment in muscular pains.[7] Studies show that the stem bark extracts of *A. polystachya* has significant *in vitro* antibacterial, mild antifungal, cytotoxic and antioxidant effects.[6,7]

MATERIALS AND METHODS

Plant material: Fresh Stem Bark of *Aphanamixis polystachya* collected from Kolhapure District, Maharashtra. The fresh stem bark were were shade dried and ground into powder with the aid of blender and stored in air tight bottles at room temperature till use.

In vitro antioxidant activity of *aphanamixis polystachya (wall.)Parker*

The *in vitro* scavenging activities of the (ME) extracts of *Aphanamixis polystachya* stem bark against different free radicals were performed. The results are expressed in terms of IC50, which is the concentration of the sample required to cause 50% inhibition of free radicals. Ascorbic acid was used as standard and the experiments were performed in triplicate

A) Assay of Nitric oxide scavenging activity

The Nitric Oxide Radical Scavenging Activity of extract was determined using the method of Sreejayan and Rao (1997) with minor modifications. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the Griess reagent. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of methanolic extract of *T. divaricata* dissolved in methanol and incubated at room temperature for 150 min. The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the incubation period, 0.5ml of Griess reagent (1% sulfanilamide, 2%

H3PO4 and 0.1% N-(1-naphthyl) ethylene-diamine-dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm (Sreejayan and Rao, 1997).[10,11]

Preparation of reagents

The extracts solutions were prepared in ethanol and the standard ascorbic acid solutions were prepared in water.

1. Sodium nitroprusside: 10 mM solution of sodium nitroprusside was prepared in distilled water.

2. Naphthyl ethylene diamine dihydrochloride (NEDD, 0.1%): 0.1g of NEDD was dissolved in 60 ml of 50% glacial acetic acid by heating and then diluted to 100 ml with distilled water. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations of (ME) extract of dissolved in methanol The Aphanamixi polystachya reaction mixture (6 ml) containing sodium nitroprusside (10mM, 4 ml), PBS (pH 7.4, 1 ml) and (ME) extract or standard solution (1 ml) were incubated at room temperature for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed and incubated at room temperature for 150 min. The same reaction mixture without the (ccl_4) extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5ml of Griess reagent (1% sulfanilamide, 2% H3PO4 and 0.1% N-(1-naphthyl) ethylene-diamine-dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm. Result shown in table 1 and figure 1.

$$Nitric\ Oxide\ Scavenges(\%) = Acontrol - Atest \div Acontrol \times 100$$

Where,

A control= Absorbance of control reaction and

A test= Absorbance in the presence of the samples of extracts.

B) Reducing power test

The reducing power of the extract was determined according to the method of Oyaizu (Oyaizu, 1986).[10-12] Different amounts of (ME) extracts (50-300mcg/ml) was dissolved in ethanol were mixed with phosphate buffer (2.5ml, 0.2mol/l, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged (650 x g at room temperature) for 10 min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and FeCl3 (0.5ml, 0.1%), and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power. Results shown in table 2 ad figure 2.

Reducing power

Reduction ability of the extract has been investigated from the Fe+++ to Fe++ transformation using the method followed by Oyaizu (Oyaizu, 1986). Earlier authors (Tanaka et al. 1988; Duh, 1998) have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh, 1998) which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation.

C) Scavenging of hydrogen peroxide

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. In this method, when an antioxidant is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide is measured spectrophotometrically. Hydrogen peroxide is a weak oxidizing agent which inactivates enzymes by oxidation of the essential thiol (SH-) groups. It rapidly transverses cell membranes and once inside the cell interior, interacts with Fe2+ and Cu2+ to form hydroxyl radicals, which is harmful to the cell.[11]

A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) and concentration was determined spectrophotometrically at 230 nm (Schimadzu UV-Vis 1700). concentrations of (ME) extract of *Aphanamixis polystachya* dissolved in Methanol (25-300 µg/ml) dissolved in methanol was added to a hydrogen peroxide solution (0.6 ml, 40mM) and the absorbance of hydrogen peroxide at 230 nm was determined after 19 min against a blank solution in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of (ME) extract and standard compounds was calculated using the following equation:

$$\%\ Scavenged(H2O2) = [\frac{Ao - A1}{Ao}] \times 100$$

Where

A0 =was the absorbance of the control, and A1=was the absorbance of EEAS or standards. Results shown in table no.3 and fig.no.3

In-vitro* anticancer activity *of aphanamixis polystachya (wall.) Parker

A) Antimitotic activity

***Allium cepa* Root Tip Meristem Model**

Locally available onion bulbs (*Allium cepa* 50 ± 10 g) were grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm length. The base of each of the bulbs were suspended on the extract inside 100 ml beakers, root length (newly appearing roots not included) and root number at 0,48, 96 hrs for each concentration of extract and control was measured. The percentage root growth inhibition after treating N-Hexane fraction of methanolic extract at 48 and 96 hrs was determined. Vincristine (standard) as well as extract of roots was used at 10

mg/ml concentration. The extract of Aphanamixis polystachya stem bark produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of extract and standard produced a growth retarding effect that was associated with a decrease in the root number.[12] Table 4.

B) Cytotoxicity study by SRB assay

Principle

Sulphorodamine B (SRB) is a bright pink Aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude.

Procedure

The monolayer cell culture was trypsinised and the cell count was adjusted to 0.5-1.0 x 105 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtiter plates, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microtiter plates. The plates were then incubated at 37°C for 72 hours in 5% CO2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4°C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using micro plate reader at a wavelength of 540nm. Table 5 The percentage growth inhibition was calculated using following formula;

$$\%\ Cell\ inhibition = 100 - \{\frac{At - Ab}{Ac - Ab}\} \times 100$$

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

RESULTS AND DISCUSSION

To the best of our knowledge we are the first which exploring the anticancer, antioxidant properties of this plant. Cancer is a disease recognised by seven hallmarks unlimited growth of abnormal cells, self sufficiency in growth signals, insensitivity to growth inhibitors, evasion of apoptosis, sustained angiogenesis, inflammatory microenvironment and eventually tissue invasion and metastasis A survey of the literature revealed that no studies on the anticancer activity of extracts of Stem bark of plants was carried out hence above mentioned, had been undertaken on the two human cancer cell lines. It is known that different cell lines might exhibit different sensitivities towards an anticancer compound, so the use of more than one cell line is therefore considered necessary in the detection of anticancer compounds. From the data obtained, it was observed Methanolic extract and it's N-Hexane fraction is effective against HeLa and MCF-7 where as extract have moderate activity comparable to the standards adriamycin.

Table 1: Results of Nitric oxide scavenging activity of extracts

Extract	Concentration (mcg/ ml)and %inhibition						IC50 (mcg/ml)
	10	20	40	60	80	100	
(ME) Extract	7.33	35.72	54.53	65.48	71.69	91.19	46.34
Std (ascorbic acid)	21.36	35.90	46.34	59.22	78.91	90.88	38.61

Table 2: Results of reducing power method

Extract	Concentration (mcg/ ml) and % inhibition					
	10	20	40	60	80	100
(ME)Extract	0.127	0.245	0.293	0.304	0.410	0.544
Std (ascorbic acid	0.109	0.206	0.315	0.466	0.564	0.623

Table 3: Results of H_2O_2 scavenging activity of extracts

Extract	Concentration (mcg/ ml) and % inhibition						IC50 (mcg/ml)
	10	20	40	60	80	100	
(ME)Extract	8.63	17.30	20.63	29.60	31.79	36.23	95.32
Std (ascorbic acid	10.70	16.65	20.92	32.92	41.46	51.32	98.97

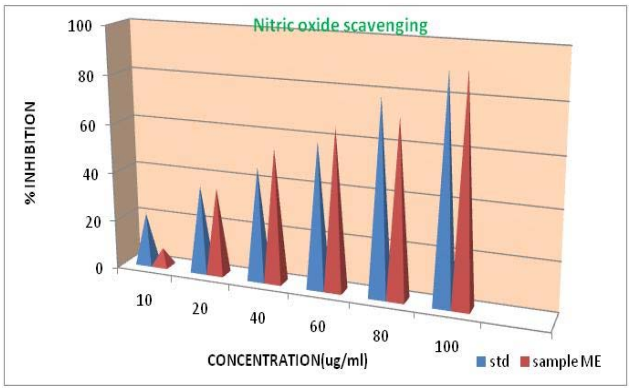

Figure 1: *In vitro* antioxidant activity of (ME) extract by Nitric Oxide method

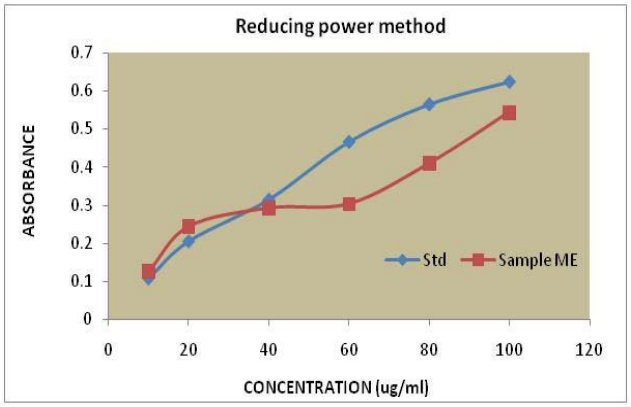

Figure 2: *In vitro* antioxidant activity of (ME) extract by reducing power method

Table 4: Observations for *Allium cepa* root length and root number attained following incubation with N-Hexane fraction of methanolic extract of *Aphanamixis polystachya stem bark* and standard drugs.

Groups	Concentration	Roots length in (cm)		
		0 hr	48 hr	96 hr
Control (Water)	--	3.37 (n=16)	4.12 (n=22)	4.63 (n=28)
N-Hexane fraction	10 mg/ml	2.42 (n=16)	2.28 (n=14)	2.15 (n=11)
Std (vincristine)	0.5mg/ml	3.87 (n=16)	3.54 (n=12)	1.97 (n=08)

Table 5: Cytotoxicity study by SRB assay

GI50 value of Sulforhodamine B (SRB) assay on different cell lines

Tissue of origin	Cell lines	Concentration (mcg/ mL)		
		ME extract	N-Hexane fraction	Adriamycin
Human Cervix	HeLa	38.5	40.0	<10
Human Breast	MCF-7	≥80	61.5	<10

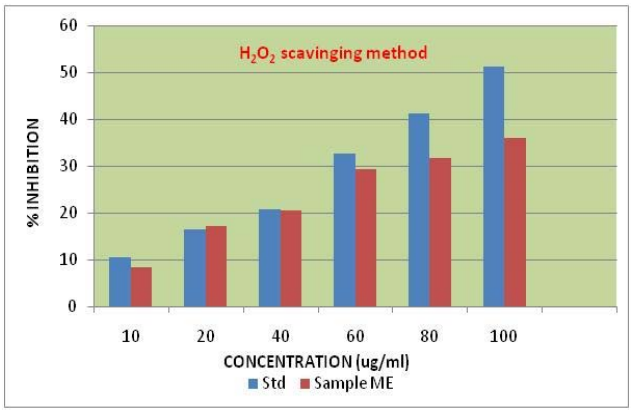

Figure 3: vitro antioxidant activity of (ME) extract by H_2O_2 scavenging activity

Note: The in vitro testing for Anticancer Activity in cell lines were tested in Tata Memorial Advanced Centre for Treatment, Research and Education in Cancer (Actrec).

All GI50 values are averages of 3 experiments.

Compound with GI50 values of ≤ 10^-6 (i.e. 1 µmole) or ≤ 10µg/ml is considered to demonstrate activity in case of pure compounds. For extracts, GI50 value ≤ 40µg/ml is considered to demonstrate activity.

Growth inhibition of 50 % (GI50) calculated from [(Ti-Tz)/(C-Tz)] x 100 = 50, drug concentration resulting in a 50% reduction in the net protein increase.

Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50% cells following treatment is calculated from [(Ti-Tz)/Tz] x 100 = -50.

CONCLUSION

Methanlic extract and its N-Hexane fraction of Aphanamixis *polystachya* stem bark powder shows most promising anticancer activity on HeLa cell line but moderate activity on MCF7.

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