Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(7); 1029-1034

ISSN: 0975-4873

Research Article

Antioxidant, Antimicrobial and Cytotoxic Activities of Alcoholic Leaves Extracts of *Spatholobus parviflorus* (Roxb.Ex Dc.) Kuntze

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Received 23rd March, 17; Revised 28th June, 17, Accepted 14th July, 17; Available Online 25th July, 2017

ABSTRACT

Objective: To investigate the total phenolics, antioxidant , antimicrobial and cytotoxic, activities of ethanolic and methanolic leaves extracts of *Spatholobus parviflorus* (Roxb.ex Dc.) kuntze. Methods: The current study was focussed on broad parameters namely total phenolics, phytochemical analysis, gas chromatography-mass spectrometry analysis and antioxidant properties in order to characterize the alcoholic extracts of *Spatholobus parviflorus* as apotential free radical quencher. Results: The phytochemical screening of alcoholic extracts of *Spaholobus parviflorus* showed the presence of various secondary metabolites. *Spatholobs parviflorus* was proved to be an effective radical scavenger in all antioxidant assays. The gas chromatographic- mass spectrometry analysis confirmed the presence of bioactive compounds in varying percentage. Conclusion: These results suggest that the ethanolic extract of *Spaholobus parviflorus* has potential of antioxidant,antibacterial and cytotoxic activity that support the ethnopharmacological uses of this plant.The remarkable activity showed by the plant extract could be attributed to the synergic effect of the active compounds present in it.

Keywords: total phenolics, cytotoxic activity, antioxidant, antimicrobial activity, *Spatholobus parviflorus.*

INTRODUCTION

Medicinal plants are a source of great economic value all over the world. Nature has given us a very rich botanical wealth and large number of diverse types of plants grows in different parts of the country. Ayurveda, Unani and Siddha are systematically used nearly 1500 plants in indigenous system of medicine. Medicinal plants are the oldest existing complete medical system in the world. Use of herbal medicines in Asia represents a long history of human interactions with the environment. Plants used for traditional medicines contain a wide ratio of substances that can be used to treat chronic as well as communicable diseases[1]. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. According to World Health Organization (WHO) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Medicinal plants contain large varieties of chemical substances which possess important therapeutic properties that can be utilized in the treatment of human diseases. The studies of medicinal plants used in folklore remedies have attracted the attention of many scientists in finding solution to the problems of multiple resistances to the existing synthetic antibiotics. Most of the synthetic antibiotics now available in the market have major setback due to the multiple resistance developed by pathogenic micro-organisms against their drugs[2]. Modern technique and pharmacological screening procedure results new plant drugs usually find their way into modern medicines. Now a day's maximum number of plant are being screened for their possible pharmacological value. The plant kingdom still hold many plant species containing substance of medicinal value which have yet to be discovered. The medicinal value of plants lies in some chemical substances that produce a definite physiologic action on the human body. The most important of these phytocompounds of plants are alkaloids, flavonoids, tannins, terpenoids and phenolic compounds.

Spatholobus parviflorus (Roxb.ex Dc.) Kuntze (Fabaceae) is a rare endemic threatened strong woody climber which is less evaluated for its medicinal properties. These woody climbers are widely distributed in a wide geographic range from Nepal, Bhutan and India. They are mainly seen in semi-evergreen and moist deciduous forests and also in sacred groves. In Kerala leaf paste is used to treat conjuctivities[3]. In northern Thailand the leaves and stem of this species are boiled with dicranopteris and used as a liquid to apply to broken bones as an analgesis[4]. Gum extracted from the wood, fibre from the bark and oil from the seeds is reputed to have economic use in Bangladesh[5]. GC- MS analysis of ethanol extracts of leaves revealed that Lupeol(28.70%) as the major component followed by the Hexadecanoic acid, [3,7,11,15]-tetramethyl-2-hexadecen-1-ol,2-tridecnol-1-ol, phytol ,1-heptatriacotanol[6].According to the recent published reports, triterpenoid Lupeol has been shown to exhibit various pharmacological activity under in vitro and in vivo conditions. These include its antioxidant, antimicrobial and anticancer activities[7].

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The present investigation have been confirmed that the plant extracts possesses antioxidant, antimicrobial and anticancer effect. Pass studies revealed that so far here is no study pertaining pharmacological evaluation of *Spatholobus parviflorus*. Hence it is imperative to evaluate the cytotoxic, antioxidant and antimicrobial activities of the plant by using established scientific method.

MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picryl-hydrazyl (DPPH), sodium phosphate, potassium ferricyanide, ammonium molybdate, ascorbic acid, trichloroacetic acid, ferric chloride, ferrozine, EDTA, catechol, 2-thiobabiuric acid (TBA), Follin ciocalteu, 2-deoxyribose and H_2O_2 (30%). All other chemicals and solvents used were of analytical grade.

Plant Material

The fresh leaves were collected from the RET garden of St.Mary's College, Thrissur, Kerala, South India and authenticated by Dr. Meena.K.Cheruvathoor, Assistant Professor, Department of Botany, St.Mary's College, Thrissur, Kerala.

Extract Preparation

Fifty grams of the powered plant material were extracted with 300mL of ethanol and methanol as solvents using Soxhlet equipment for 24hours.

Preliminary Phytochemical analysis

The sample extracts were analysed for the presence of flavonoids, alkaloids, glycosides, steroids, phenols, saponins and tannins according to standard methods[8].

Determination of total phenolics.

The concentrations of phenolic content in ethanol and methanol fraction were determined with Folin Ciocalteu's phenol reagent (FCR). The 1 ml of the extracts was added to 8 ml of distilled water and 1 ml of FCR, and mixed thoroughly. After 15 min, 3 ml of sodium carbonate (20%) were added to the mixture and shaken for 2 h at room temperature. The absorbance was measured at 765 nm [9].The concentration of phenolic compounds was obtained from standard catechol graph.

Antioxidant properties

DPPH free radical scavenging assay

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants. Hydrogen or electron donation abilities of the compounds were measured from the bleaching of the purple-colored methanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). The sample solution of material (50 µL) at four concentrations (1.0, 0.5, 0.25 and 0.125 mg/ml) were mixed with freshly prepared methanolic solution of DPPH (634 μM) and allowed to stand for 30 min at room temperature. The absorbance was then measured at 515nm using a spectrophotometer. L-ascorbic acid was used as positive control[10].

Hydroxyl Radical Assay

The 2-deoxyribose assay was used to determine the scavenging effect of the extracts on the OH radical[11]. Each reaction mixture contained, the following final concentrations of reagents in a final volume of 1.0 ml 2-deoxyribose (2.5 µM), potassium phosphate buffer (pH 7.4, 20 mM), $FeCl_3$ (100 µM), EDTA (104 µM), H_2O_2 (1 mM), and L-ascorbic acid (100 µM). The mixtures were incubated for 1 h at 37°C, followed by addition of 1.0ml of 1% (w/v) TBA in 0.05 M NaOH and 1.0 ml of 2.8% (w/v) TCA. The resulting mixture was heated for 15 min at 100°C. After cooling on ice, absorbance was measured at 532 nm.

Ferric Reducing Ability Power

Ferric ions reducing power was measured according to the method of Oyaizu[12]. Higher absorbance of the reaction mixture indicated greater reducing power. Extracts were mixed with 1ml of 20mM phosphate buffer and 1ml potassium ferricyanide (1%, w/v)and incubated at 50°C for 30 min. 1ml of TCA(10%, w/v) and 0.5ml ferric chloride (0.1%, w/v) were added to the reaction mixture and absorbance was measure at 700nm.

Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

Cupric ion reducing capacity was measured in accordance to the method of Apak[13]. 1ml 10mM cupric chloride ($CuCl_2$), 1ml 7.5mM neocuprione and 1ml 1M ammonium acetate buffer (pH 7) solutions were added to test tubes. The extracts were mixed with reaction mixture independently .These reaction mixture were incubated for half-hour at room temperature and measured against blank at 450nm.

Fe^{2+} chelating activity assay

The chelating activity of the extracts for ferrous ions(Fe^{2+}) was measured . To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta coloured complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm[14].

Antimicrobial Activity

Test microorganisms

The microorganisms used for antibacterial evaluation such as *Bacillus cereus* (MTCC-1305), *Enterobacter faecalis* (MTCC-5112), *Salmonella paratyphi*, (MTCC-735), *Staphylococcus aureus* (MTCC-96), *Escherichia coli* (MTCC-729)

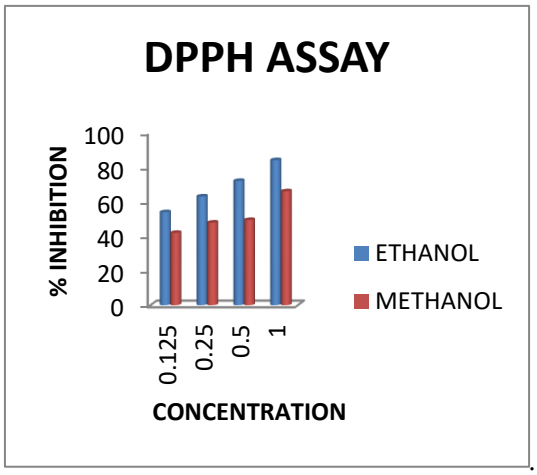
were obtained from Microbial Type Culture Collection and gene bank (IMTECH, Chandigarh, India).

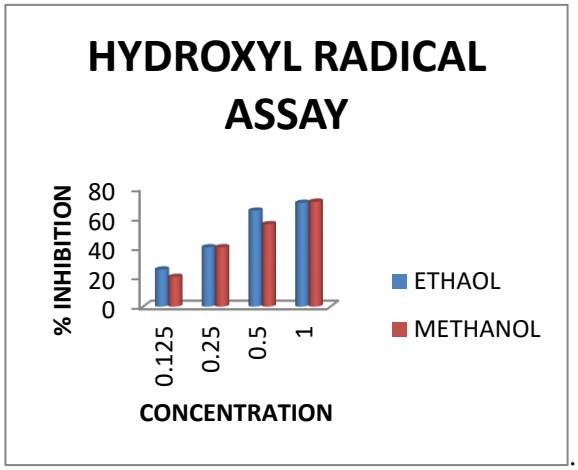
Antimicrobial activity assay

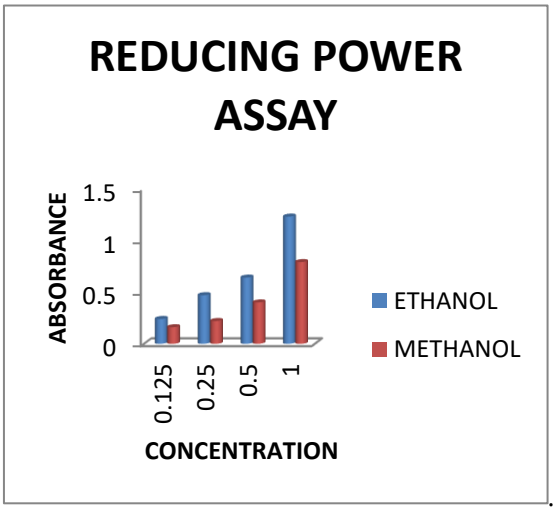
The agar diffusion method is used for the antimicrobial evaluations. Wells of 8mm diameter were dug on the inoculated nutrient agar medium (antibacterial assay) with sterile cork borer and 50µl of the extracts of *Spatholobus parviflorus* was added in each well. Wells introduced with 50µl of pure ethanol and methanol served as negative control. The plates were incubated at 37^{0C} over night and examined for the zone of inhibition. The diameter of the inhibition zone was measured in mm. The standard antibiotic drug ciprofloxacin was used for antibacterial evaluation and standard the diameter of the inhibition was equal to or larger than 8mm .

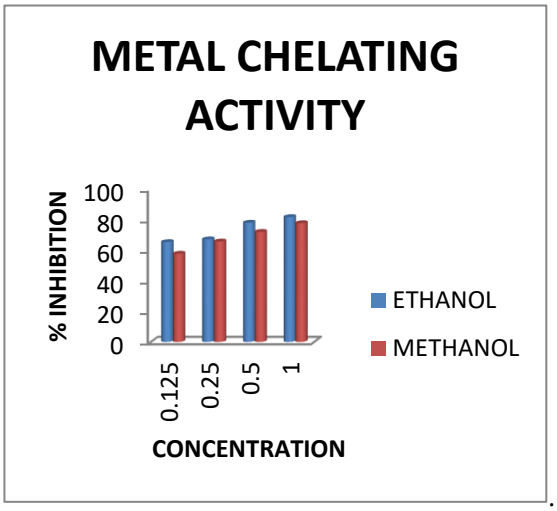
Cytotoxic Assay

Cell lines









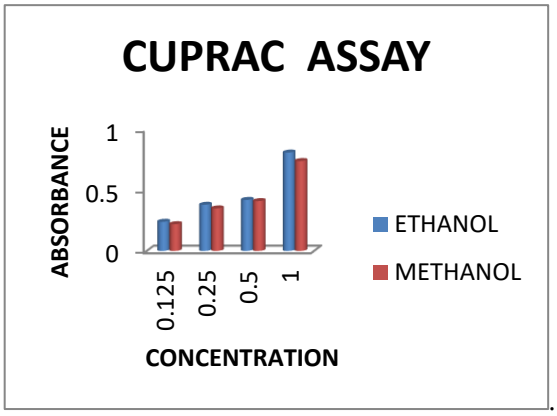


Figure 1: Results of antioxidant activity from different assays.

Dalton's Lymphoma Ascites [DLA] cells were being maintained in intraperitoneal cavity of mice in Amala Cancer Research Centre, Thrissur, Kerala.

Cytotoxic activity

The extracts were studied for short term in vitro cytotoxicity using DLA cells using trypan blue dye exclusion technique. The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS. Cell viability was determined by trypan blue exclusion method. Viable cell suspension ($1x10^6$ cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 ml using PBS. Control tube contained only cell suspension. These assay mixture were incubated for 3 hour at 37^0C. Further cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of tyrpan blue while live cells do not take up the dye. The number of stained and unstained cells was counted

Table 1: Inhibition Zones formed by *Spaholobus Parviflorus* leaf extract.

Diameter of inhibition zones(mm/50µL)				
Microorganisams	Standard antibiotics (ciprofloxacin)	ethanol extract	methanol extract	control
Bacillus cereus	27	25	24	---
Enterobacter faecalis	25	22	20	---
Salmonella paratyphi	29	25	23	---
Staphylococcus aureus	25	21	18	----
Escherichia coli	28	20	17	---

separately. Control tube contains only one dead cell. The sample dissolves in DMSO.

% cytotoxicity = { [No. of dead cells / (No. of live cells + No. of dead cells)] X100 }

2.7 GC-MS analysis

GC-MS analysis was carried out at CSIR-NIIST,Govt.of India. Thiruvananthapuram, Kerala. Analysis was carried out on a GC, Perkin Elmer, and XLGL system. Identification of bioactive compounds using database of National Institute Standard and technology (NIST) is having more than 62,000 patterns.

RESULTS AND DISCUSSIONS

Preliminary phytochemical screening of extract

Phytochemical evaluation was performed with ethanol and methanol extracts of *Spaholobus Parviflorus* leaves. Both extracts showed the presence of higher number of phytochemicals. Ethanol extract was found to be rich in Phenols, Flavonoids, Cardiac glycosides, Steroids, Terpenoids, Saponins, Xanthoprotein, quinine, Coumarin and Anthraquinone than methanol extract.

Determination of total phenolics

Total phenol content of ethanol & methanol extracts of *Spatholobus Parviflorus* are 0.485±0.162 & 0.470±0.2 mg catechol equivalent per gram of plant extract respectively. It revealed that ethanol extract contains more phenolic components and has higher antioxidant activity and act as free radical scavengers.

Antioxidant properties

DPPH Assay

Figure (1) shows a decrease in the concentration of DPPH radical due to the scavenging ability of the soluble constituents in the ethanolic and methanolic leaves extracts of *Spaholobus parviflorus*.There was a direct positive relationship between antioxidant activity and increasing concentration of the extracts[17]. A higher antioxidant power shown by the ethanol extract [IC_{50} 51.44µg/ml] as compared to methanolic extract [IC_{50} 63.27µg/ml].

The Hydroxyl Radical Scavenging Assay

The most important reactions of free radicals in aerobic cells involved molecular oxygen and its radical derivatives, peroxides and transition metals. Reactive oxygen species are thought to play an important role in aging and chronic diseases[18]. The study showed that both extracts showed remarkable antioxidant activity (figure 1) on hydroxyl radical. The IC_{50} values of the scavenging hydroxyl radical activity was 86.04µg/ml and 86.98µg/ml for ethanol and methanol extracts respectively.

Ferric Reducing Ability Power

Reducing power method for the determination of antioxidant activity determines the capacity of reducing the oxidation potential of oxidants. The reducing power of the samples due to the hydrogen donating abilities. In this assay ascorbic acid was used as standard. Increased absorbane of the reaction mixture indicates increase in reducing power[19]. Out of two extract studied, ethanol extract showed good antioxidant activity (figure 1).

Cupric Ion Reducing Antioxidant Capacity

The CUPRAC assay utilizes Cu (11)-neocuprine reagent as the chromogenic agent. It is based on the measurement of absorbance at 450 nm by the formation of stable complex between Cu(11) and neocuprine. The cupric ion

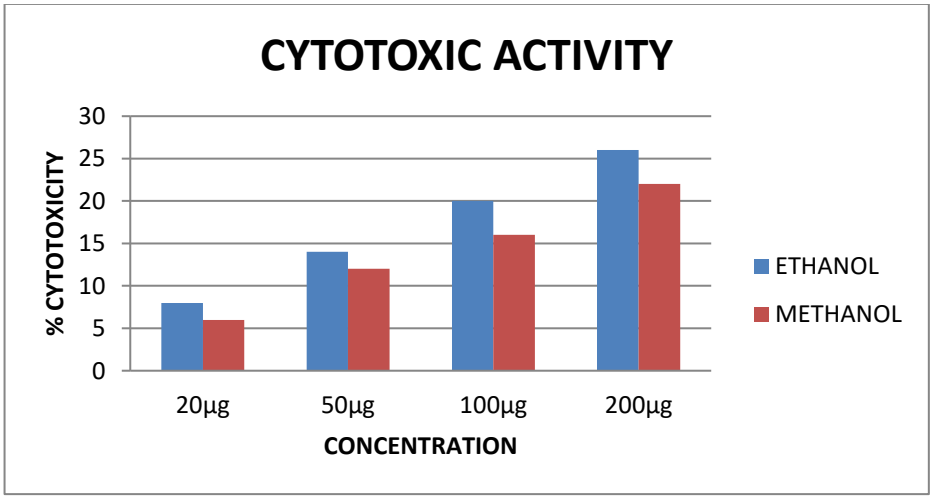


Figure 2: The cytotoxic activity of ethanol & methanol extracts.

Table 2: Phyto components identified in methanol leaf extract of *Spatholobus parviflorus.*

RT	Name of compound	MF	MW	Percentage
5.376	Benzene,methoxy	C_7H_8O	108	2.95
7.780	Benzene, 1,3-dichloro	$C_6H_4Cl_2$	147	4.90
9.476	Benzoic amide,2-acetoxy-N-methyl	$C_{10}H_{11}NO_3$	193	3.97
12.450	Naphthalene	$C_{10}H_8$	128	3.82
15.505	Cyclohexasiloxane,dodecamethyl-	$C_{12}H_{36}O_6S$	444	2.50
20.055	Cycloheptasiloxane,tetradecamethyl	$C_{14}H_{42}O_7S$	518	4.77
20.480	Tetradecane	$C_{14}H_{30}$	198	3.24
28.513	Phytol	$C_6H_{40}O$	296	2.86
30.339	Hexadcanoic acid,methyl ester	$C_{17}H_{30}O_2$	266	5.23

reducing ability of extracts is shown in figure (1). Cu^{2+} reducing capability measured by this method was found to be concentration dependent. Ethanol extract shows high reducing power.

Fe^{2+} Chelating Activity Assay

In this assay the antioxidative action is carried out by chelation of transition metal. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the co-existing chelator can be determined by measuring the rate of color reduction. The chelating effct of the extract on ferrous ions is shown in figure (1).It is observed that inhibition percentage values go on increasing with continuous increase in concentration of plant extracts in the assay mixture. The IC_{50} values of chelating ability was found to be 44.91µg/ml and 51.51µg/ml for ethanol and methanol extracts respectively.

Antimicrobial screening

The antibacterial screening of the leaf ethanol extract of *Spatholobus Parviflorus* showing the zone of inhibition in millimetres, for Gram positive and Gram negative bacteria is summarized in Table 1.

Invitrocytotoxic activity

The cytotoxic activity of ethanol and methanol extracts were shown in the figure (2).The ethanol extract manifested a strong cytotoxic activity against target cells in vitro. The cyclophosphamide had taken as positive control.

GC-MS analysis

The compounds present in methanol extract of leaf of *Spatholobus parviflorus* was identified by GC-MS analysis. The active principle with their molecular formula,molecular weight, retention time and percentage are presented in table 2. Nine compounds were identified from methanol extract. The result revealed that Hexadcanoic acid,methyl ester [5.23%] as the major component followed by Cycloheptasiloxane, tetradecamethyl, Phytol. The major constituents hexadcanoic acid,methyl ester and phytol were said to possess various activities such as antioxidant,antimicrobial and anticancer properties[20]. GC-MS analysis of ethanol extract was already reported[6] and the results reveal that lupeol [28.70%] as the major component followed by the hexadeanoic acid. It is very clear that the major compound is a well known antioxidant,antimicrobial and anticancer agent[21].

CONLUSION

It can be stated that ethanol leaves extracts have a strong antioxidant, antimicrobial and anticancer activities compared to methanol extract. On basis of these results, *Spatholobus parviflorus* appear to be good and safe natural antioxidant, antimicrobial and anticancer agents and also, could be of significance in human therapy, animal and plant diseases. Further studies should be done to search new compounds from *Spatholobus parviflorus.*

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

AKNOWLEDGMENT

The authors are grateful to Principal, Vimala College Thrissur and little flower college Guruvayur, Director Amala cancer research centre Thrissur, Kerala, for encouragement and providing facilities for doing this research work.

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