World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

EXPLORATION OF CYTOTOXIC AND ANTIOXIDANT POTENTIAL OF *PONGAMIA PINNATA (FAMILY: FABACEAE)*

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Article Received on 10/11/2022 **Article Revised on 30/11/2022** **Article Accepted on 21/12/2022**

ABSTRACT

Leaves of *Pongamia pinnata* commonly known as 'karanj' were explored for their antioxidant and cytotoxic potential. Four extracts were prepared on polarity basis these were hexane, chloroform, acetone and methanol extracts. Phytochemical analysis of fresh leaves and extracts revealed the presence of phytoconstituents like flavonoids, tannin, sapponins and terpenoids. Methanol extract exhibited significant antioxidant potential in DPPH radical scavenging assay with 93.3% inhibition. In case of FRAP assay however, best activity was revealed by Chloroform extract. Cytotoxic potential of acetone and chloroform extract revealed more than 75% activity against brine shrimp larvae.

KEYWORDS: *Pongamia pinnata*, DPPH, FRAP, Antioxidant, Cytotoxic, Brine shrimp assay.

INTRODUCTION

Pongamia pinnata belongs to family Fabaceae, is a medium height tree commonly known as Indian beech and Karanj.[1] Tree is mainly known for the oil production as same is used as biodiesel and in soaps, varnishes and as insect repellent.[2] Many parts of tree are used in ethnomedicine.[3] Bark of the tree is useful as anthelmintic, haemorrhoids, beriberi, opthalmopathy and ulcers.[4] Juice of leaves are used for cold, cough, diarrhoea, dyspepsia, flatulence, gonorrhoea and leprosy.[5] Although plant is well known for its medicinal potential but as it is growing under forest canopy in the germplasm garden of Regional Plant Resource Centre under shade, thus was explored for its cytotoxic, antioxidant and phytochemical potential in order to understand, if its medicinal properties are reduced or even under such adverse conditions plant remains medicinally useful.

MATERIALS AND METHODS

Collection and processing of plant material

Fresh leaves of medicinal plant *(Pongamia pinnata*) were collected from the medicinal germplasm garden of Regional Plant Resource Centre (RPRC), Bhubaneswar. The leaves were dried in shade. After drying, the leaves material were grinded using mechanical blender(*Lexus make*) in to fine powder and transferred in to airtight containers with proper labelling for future use.

Moisture content determination.

Moisture content of the plant was determined by comparing the weights of fresh and dried leaves. Moisture content was determined by the formula.

Moisture content = **Fw-Dw / Dw × 100**

Where, Fw = fresh weight of leaves samples

And Dw = dry weight of powder samples

Extraction of the plant sample

Solvent extracts were prepared by Maceration extraction methods. About 90gm of powder plant material was taken in a beaker (1litre) and extracted with 500ml of solvents from non polar to polar solvents. Solvents used were hexane, chloroform, acetone and methanol. The process of extraction was repeated for 3 times. After extraction, extracts were concentrated under vaccum at lower temperature of 40 ^{0}C using Rotary Evaporator (Bucci make). Concentrated extract were kept in tight screw cap vials and were stored in a refrigerator at 4^{0}C till further use.

Phytochemical analysis

Phytochemical tests were conducted following the standard protocols.[6] Methodology of the same was as follows:

1. Test for Alkaloids: Alkaloid tests were done by using 3 different reagents.

- **Dragendroff's test -** To 1ml of extract 2ml of 1% HCl was added and then boiled for few minutes, after boiling 2-3 drops of Dragendroff's reagent was

added & sample was observed for reddish brown precipitate.

- **Wagner's test -** To 1ml of extract 1ml of 1% H_2SO_4 was added followed by few drops of Wagner's reagent. Formation of precipitate depicts the presence of alkaloids.
- **Mayer's test -** To 1ml of extract, 2ml of 1% HCl and Mayer's reagent was added drop wise and was observed for the formation of precipitate.

2. Test for Flavonoid: To 2.5 ml of extract, 1 ml of 10% NaOH was added. From the side of the test tube, drops of conc. HCl were added. Yellow colour turns to colourless which indicates presence of flavonoids.

3. Test for Anthraquinone: To 1ml of extract 2ml of 5% of KOH was added and was observed for pink colouration.

4. Test for Saponin: To 1ml of extract 2ml of $NaHCO_3$ was added and on shaking forms lather.

5. Test for Terpenoids: To 1ml of extract 400µl of chloroform and 4-5 drops of conc.H_2SO_4 was added from the walls of the test tube. A reddish brown ring indicates the presence of terpenoids.

6. Test for Cardiac glycoside: To 2.5ml of extract 2ml of glacial acetic acid**,** few drops of $FeCl_3$ and H_2SO_4 was added from the walls of the test tube. Presence of cardiac glycoside is determined by reddish brown ring.

7. Test for Tannin: 1ml of extract was boiled and few drops of $FeCl_3$ were added to it. The sample was observed for blue, black, green colour.

8. Test for Starch: To 1ml of extract 500µl of iodine was added, which results in blue coloration.

9. Test for Phlobatannin: To 1ml of extract 1% HCl was added and boiled, formation of precipitate occurs on positive test.

Cytotoxic activity

Brine shrimp (Artemia salina) mortality assay

Cytotoxic activity study was carried out by brine shrimp lethality assay using standard protocol[7] Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Brine shrimp (*Artemia salina*) eggs were hatched in artificial sea water, which was prepared using black salt 3.6 gm/ 200 ml distilled water. The eggs were incubated for 48 hours at temperature of about 28° C to get the desired growth of the larvae for biological evaluation. For each dose level 3 replicates were used. To each test tube of negative control, positive control and extracts, 20 numbers of brine shrimp larvae were taken and volume was made up to 10ml by adding salt water. Cytotoxic assay was carried out at four doses 25, 50, 100 and 200µg/ml. Motility assessment of larvae was conducted at each hour up to four hours.

Motility readings were graded as below

4+ = high motile, 3+ = motile, 2+ = sluggish, 1+ = slow, Nil = no activity

After 24 hrs, the number of survived larvae in the control and experimental tubes were counted. From this data, the percentage (%) of inhibition of the brine shrimp was calculated for each concentration using the following formula.

Percentage Inhibition =

$$\frac{\text{No of larvae in control} - \text{No of larvae in experimental}}{\text{No of larvae in control}} \times 100$$

Antioxidant activity

Qualitative Analysis (TLC based antioxidant assay)

TLC is one of the most widely used and potent technique to resolve mixture of plant compounds. It is also called DPPH (2,2-Diphenyl 1-picrylhydrazyl) assay. TLC sheet (Silica gel 60 F_{254}, Merck company, Germany) was used as stationary phase. The developed TLC plate was sprayed with 0.2% DPPH in methanol as an indicator as per the standard protocol.[8]

The presence of antioxidant compounds were detected by yellow spots against a purple background on the TLC sheet sprayed with 0.2% DPPH in methanol.

DPPH+AH ——› DPPH–H+A^-

(Violet color) (Yellow color)

Three types of solvents were prepared for TLC chromatography technique.

1. BEA – Benzene: Ethanol: Ammonium hydroxide (90:10:1) [Non polar/Basic]

2. CEF – Chloroform: Ethyl acetate: Formic acid(5:4:1) [Intermediate polarity/Acidic]

3. EMW – Ethyl acetate: Methanol: Water(40:4.5:4) [Polar/Neutral]

Qualitative screening of the constituents in each of the plant extract of *Pongamia pinnata* for antioxidant activity was done by TLC analysis. The precoated TLC sheets were activated at 100^0C for 2 minutes. The samples were spotted with the help of micro tips leaving 1cm from the bottom of the sheet. After drying of sheets DPPH solution was sprayed. Yellow bands on purple background represent the antioxidant bands of the extract.

Rf values of all the antioxidant bands were calculated using the following formula.

Retardation factor (R_f) =

$$\frac{\text{Distance travelled by the compound}}{\text{Total Distance travelled by the solvent}}$$

Quantitative anti-oxidant Analysis

Quantitative analysis was done by two popular methods as follows.

DPPH free radical scavenging assay

For DPPH free radical scavenging assay 1mM DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared by adding 4mg of DPPH dissolved in 10ml methanol. DPPH assay was done by serial dilution method starting from concentration of plant extracts (7.8 µg/ml, 15.62 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500µg/ml, 1000µg/ml) was prepared in methanol. 1ml of

each sample was taken in the test tubes and 500µl of DPPH solution was added. For control, each test tube contained 1ml methanol and 500µl DPPH**.** Samples were incubated for 30 minutes at room temperature in dark. All the samples were taken in triplicate and complete set of experiment was repeated three times. Optical density (OD) was measured at 517nm in spectrophotometer.[9] The percentage of free radical scavenging activity was calculated from the following formula.

Percentage free radical scavenging [DPPH] = [(Ac – As) ÷ Ac] × 100

Where, Ac = Absorbance of control and As = Absorbance of sample.

FRAP ASSAY (Estimation of total antioxidant activity)

Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay as per the standard protocols.[10] Spectroscopic method is based upon the ability of antioxidants to reduce Fe^{+3} to Fe^{+2} in the presence of TPTZ, forming an intense blue Fe^{+2}-TPTZ complex with an absorption maximum at 593 nm. The decrease in absorbance is proportional to the antioxidant present. The FRAP reagent (300 mM Acetate buffer pH 3.6: 40 mM Dilute HCl: 10 mM TPTZ: 20 mM $FeCl_3.6H_2O$ in the ratio of 10: 1: 1) was prepared and then incubated at 37^oC in a water bath for 10 minutes. Absorbance of FRAP reagent was taken at 0th minute (t_0) which was the control of the experiment. Ascorbic acid was taken as standard. A total of 100 µL of sample/standard and 300µl of distilled H_2O was then added to the FRAP reagent and incubated at 37°C for 4mins (t4). A reagent blank was prepared as described above but 100µl of distilled H2O was added instead of test sample. Duplicate test tubes were taken and absorbance was measured at 593nm. Ascorbic acid was taken as standard and 1.0mM to 0.1mM concentration of standard was prepared for the FRAP assay and based on the observations a standard curve was plotted. A number of dilutions of each sample extract were tested allowing dose response curves to be produced. The FRAP values were expressed in mmol Ascorbic acid equivalents (AAE).

RESULTS AND DISCUSSION

Moisture content of the leaves of *Pongamia pinnata* was found to be 54% and as can be observed from Table 1 highest yield was obtained of methanol extract followed by chloroform, acetone, hexane respectively. Thus polar molecules outnumber the non polar ones.

Table 1: Percentage of yield of solvent extracts.

Extract	Weight of sample	Yield
Hexane	0.947	1.05%
Chloroform	1.419	1.57%
Acetone	1.126	1.25%
Methanol	1.578	1.75%

Phytochemical analysis of leaves of Pongamia pinnate

As can be observed from the Table 2, Fresh leaves showed the presence of Tannin, Cardiac glycoside and Terpenoids. Presence of terpenoids and cardiac glycosides is in confirmation with the other studies.[11] However, tannin was not reported earlier.

Table 2: Phytochemical analysis of fresh leaves of *Pongamia pinnata.*

Phytoconstituent	Results
Alkaloid	-
Flavonoid	-
Anthraquinone	-
Tannin	+
Cardiac glycoside	+
Terpenoids	+
Saponins	-
Phlobatannin	-
Starch	-

(+)Presence of phytoconstituent, (-)absence of phytoconstituent

Brine shrimp mortality assay (cytotoxicity)

Artemia salina is one of the convenient organisms for toxicity tests in *vitro* due to its robust nature and easy maintenance in the laboratory condition.[12] The bioassay study with Artemia larvae was carried out as short term toxicity test and only on freshly hatched nauplii. As can be seen in Fig1 All the extracts showed dose dependent activity. Acetone extract showed highest activity amongst all the extracts which was 86.26 % at the higher dose of 200microgram/ml followed by choloroform extract(77.08%). As brine shrimp assay has a correlation with anticancer activity[13] hence acetone extract and chloroform extracts have provided lead for further exploration.

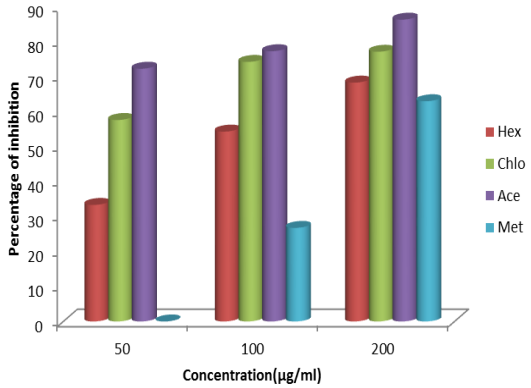


Fig 1: Cytotoxic activity of of *Pongamia pinnata.*

Antioxidant activity of Pongamia pinnata

Qualitative antioxidant screening

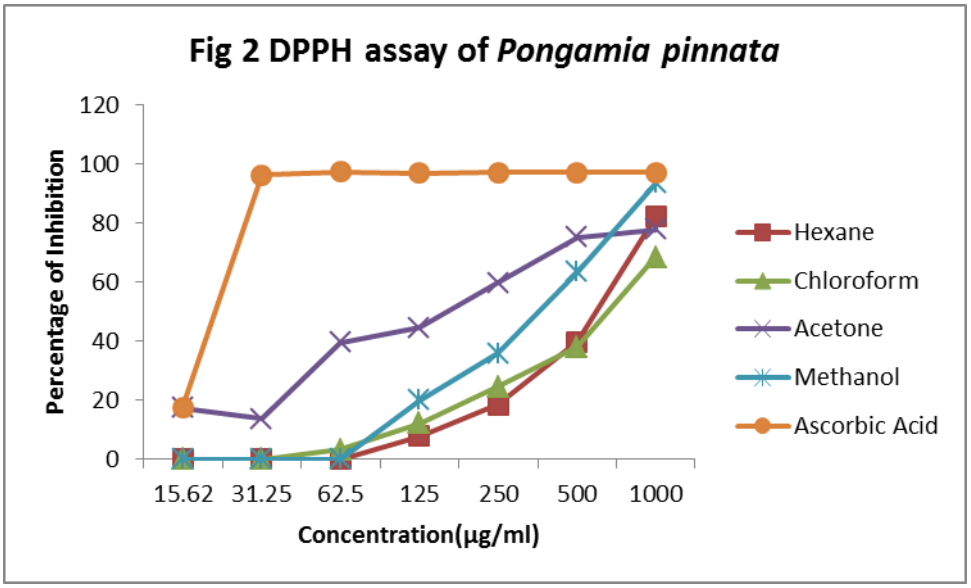
Qualitative antioxidant activity was conducted using TLC based DPPH assay, Best separation was obtained in BEA solvent where all the extracts showed maximum number of antioxidant bands with maximum number of 10 antioxidant bands in the Chloroform extract.

Table3: TLC based antioxidant assay of solvent extracts of *Pongamia pinnata leaves*			
Solvent extract	**Solvent System**	**Rf Values**	No of bands
Hexane extract	BEA	0.18, 0.24, 0.29, 0.74, 0.86, 0.93	6
	CEF	0.6	1
	EMW	0.25	1
Chloroform ext.	BEA	0.07, 0.17, 0.24, 0.3, 0.43, 0.48 0.56, 0.75, 0.84,0.94	10
	CEF	0.17, 0.58, 0.71, 0.78, 0.88	5
	EMW	0.05, 0.15, 0.24, 0.36, 0.79	5
Acetone extract	BEA	0.22, 0.3, 0.4, 0.7, 0.84, 0.94	6
	CEF	0.71, 0.77	2
	EMW	No separation	0
Methanol extract	BEA	No separation	0
	CEF	No separation	0
	EMW	Streak	Infinite

Quantitative antioxidant activity

DPPH free radical scavenging assay

The antioxidant activity of different extract of *Pongamia pinnata* was analysed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of a free radical scavenger, the absorption decreased and the resulting discoloration was related to the number of elecrons gained. The DPPH free radical scavenging activity of extract (Fig 2)was less as compared to the standard Ascorbic acid, but at higher dose of 1000microgram/ml methanol extract showed equivalent activity when compared with the standard. At the same dose hexane and acetone extract also showed more than 80% activity. This suggests strong antioxidant potential of the plant.



Ferric reducing anti-oxidant power assay

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of $Fe^{3+}$to Fe^{2+}.The product was visualized by forming the intense Prussian blue color complex and then measured at λ_{593} nm. All the extracts showed mild activity in FRAP assay.

Table 5: FRAP values of extracts of *Pongamia pinnata*	
Extract	**mM of Ascorbic acid equivalent**
Hexane	0.498±0.333
Chloroform	0.393±0.183
Acetone	0.958±0.083
Methanol	0.661±0.011

ACKNOWLEDGEMENTS

Authors acknowledge the Forest, Environment and Climate change Department, Government of Odisha for providing funding for the smooth conduct of entire work and also acknowledge Chief Executive, Regional Plant Resource Center, Bhubaneswar, Odisha for providing lab facilities.

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