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ANTIMICROBIAL ACTIVITY OF HYDRO-ALCOHOLIC BARK EXTRACT OF *PONGAMIA PINNATA*

P. Niharika*, G. Radhika, A. Kanthi Sri, Syed Fathima and Y. Elisha

Department of Pharmaceutical Chemistry, SIMS College of Pharmacy, Mangaldas Nagar, Guntur - 522001, Andhra Pradesh, India.

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Correspondence to Author:

P. Niharika

Flat no- 501, Tulasi Heights,
Adithya Gardens, Etukuru, Guntur -
522017, Andhra Pradesh, India.

E-mail: niharika.mpharm12@gmail.com

ABSTRACT: Kanuga (*Pongamia pinnata*), a plant belonging to *Leguminosae* described in Vedas has ethnobotanical importance and a variety of medicinal properties. *Pongamia pinnata*, commonly grown on the roads of India is used to heal wounds, treat many fungal infections, skin diseases and to clean teeth. The objective of the current study is to perform phytochemical analysis and appraise the antimicrobial activity of hydro-alcoholic bark extract of *Pongamia pinnata*. Dried, powdered bark was extracted with water and ethanol (1:1) mixture by soxhlation for 72 h. Following the standard tests of phytochemical investigation, the hydro-alcoholic bark extract subsumes constituents like flavonoids, phenolic compounds, carbohydrates, saponins, glycosides, and alkaloids. Minimum Inhibitory Concentration (MIC) of the hydro-alcoholic bark extract was evaluated by serial tube dilution method, and MIC for *Bacillus subtilis*, *Escherichia coli*, and *Aspergillus niger* was 100 µg/ml, 100 µg/ml, and 100 µg/ml, respectively. The antimicrobial activity of hydro-alcoholic bark extract of *Pongamia* was assessed by agar well diffusion method. Organisms used for antimicrobial assessment include *Bacillus subtilis*, *Escherichia coli*, and *Aspergillus niger*. The standard antibiotics used for antibacterial, antifungal assay were ciprofloxacin and fluconazole, respectively.

INTRODUCTION: *Pongamia pinnata* known as Hongay oil tree or Karanj or Kanuga is a member of the Fabaceae/Leguminosae family, which comes under the order Fabales. The synonyms of *Pongamia pinnata* are *Derris indica*, *Pongamia glabra*, *Millettia*. *Pongamia* is a medium-sized evergreen legume tree native to India, northern Australia, Indonesia, and Southeast Asia commonly grown on alluvial and coastal habitats from sea level to 1200m.

It grows wild in the coastal, tropical areas with a warm and humid climate and in areas with well-distributed rainfall.

It grows in almost all types of soils, highly in river banks for controlling soil erosion and binding sand dunes. This plant tolerates drought and salinity and is also used for afforestation. Propagation is by seed and root suckers^{1,2}. The leaves are soft, shiny, mature to glossy deep green, and are imparipinnate, alternate with 5-7 leaflets, ovate and opposite. Flowers are lilac or pinkish white and fragrant, in auxiliary racemes blossoming all throughout the year. The brown seed pod is compressed, woody, indehiscent, and yellowish grey when ripe. The bark is a short bole spreading crown and greyish-green or brown in color³. Chemical constituents of *Pongamia* include ponganones III – XI⁴ from its

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root bark, linoleic acid, oleic acid, palmitic acid, eicosanoic acid, demethoxy-kanugin, glabrin, glabrasaponin, kanugin, neoglabrin, pinnatin⁵, pongamol, pongaglabrone and pongapin, and kanjone from seeds⁶.

Recent review reveals the isoflavones, furrugone and 6,7-dimethoxy-3',4'-methylenedioxy-8-(dimethylallyl) isoflavone from seeds of *Millettia* or *Pongamia* reported to have potent cytotoxic effects on human ovarian cancer cell lines by inducing apoptosis⁷. A chalcone of *Pongamia* called Millepachin inhibits topoisomerase II and elevate NF-kB, thereby inducing apoptosis in the ovarian cancer cells⁸. Lonchocarpin, a chalcone from the root of *Pongamia pinnata* reduced lung cancer cell proliferation by modulating the Caspase pathway⁹. The isoflavone, Durmillone from the dried stem of *Millettia* produced apoptosis and autophagy in cancer cell lines (Hep G2, MCF-7, HeLa)¹⁰. Zinc oxide nanoparticles synthesized with seed extract of *Pongamia pinnata* have cytotoxic activity on breast adenocarcinoma cell line (MCF-7) and effectively inhibited the growth of *Bacillus licheniformis* and biofilm-forming *Candida albicans*¹¹. Robustic acid and thoningine-C, coumarins from seeds of *Pongamia* have promising antifungal activity on *Candida albicans* and were proved to be non-toxic on mammalian cells¹².

Karanjin, a furan flavonol, has gastroprotective activity and had shown anti-colitic action by decreasing the inflammatory infiltration, oedema, necrosis, epithelial destruction, segmental ulceration, and restoration of the normal architecture of the colon¹³. Karanjin and pongapin have promising nitric oxide scavenging property, and these furanoflavones were the natural alternatives to treat psoriasis without side effects¹⁴. Karanjin from seeds of *Pongamia* have shown antioxidant and neuroprotective activity¹⁵. Glabrachalcones, karanjin, and aqueous seed extract possess anti-HIV activity¹⁶. Glabarachalcone and isopongachromene from the aqueous seed extract have anti-HBV (Hepatitis B virus) activity by inhibiting the attachment of HBsAg (Hepatitis B surface Antigen) onto its receptor and showed good binding to HBV DNA polymerase *in-silico*¹⁷. Ethanolic stem extract and lanceolatin B of *Millettia* possess significant anti-inflammatory and analgesic activity¹⁸.

Hydro-alcoholic extract of twigs of *Pongamia pinnata* has anti-carries activity¹⁹. The petroleum ether extract of stem bark of *Pongamia* reduced blood glucose level, QT prolongation, QTc interval, controlled the levels of cardiac biomarkers, improved oxidative stress and proved as a potential remedy for cardiomyopathy in diabetics²⁰. The literature survey reveal that *Pongamia* possesses anticancer, cardioprotective, antihyperglycemic, antioxidant, anti-inflammatory, anti-fungal, anti-bacterial, pesticidal, larvicidal, neuroprotective activities. The present hypothesis is focused on determining the phytochemical constituents, antimicrobial activity of hydro-alcoholic extract of bark of *Pongamia pinnata*.

MATERIALS AND METHODS:

Collection and Identification of Plant: The bark of *Pongamia pinnata* was collected from premises of Mahatma Gandhi College, Guntur district, Andhra Pradesh, India, in the month of December 2016, and its authentication was confirmed by Dr. S. M. Khasim, Botany Department Acharya Nagarjuna University College of Life Sciences, Guntur. Specimen of the plant has been deposited at Department of Botany for future reference.

Plant Material: The collected plant bark was separated and cleaned with deionized water to remove extraneous matter, dried under shade for two weeks at room temperature. The dried bark of the plant was thoroughly grounded and sieved using 0.3mm mesh. The powder was stored in an airtight container and maintained at 4 °C until use.

Preparation of Extract: 250gm of powdered material of *Pongamia pinnata* bark was extracted with hydro-alcoholic solution sequentially using soxhlet apparatus for 72 h. The extract was filtered, and the filtrate was concentrated under reduced pressure using a rotary evaporator at 40 °C until the extra solvent gets evaporated. The yield of aqueous extract was found to be 70 %. The extract was stored in the refrigerator at 4 °C until further use.

Preliminary Phytochemical Screening: The extract of the powdered bark of *Pongamia pinnata* was analyzed for the presence of various phytoconstituents like steroids, triterpenoids, saponins, triterpenoid saponins, alkaloids, carbohydrates, flavonoids, glycosides, and phenolic

compounds using standard phytochemical procedures as described below.

Test for Steroidal Saponins: The extract is hydrolyzed with sulphuric acid (H_2SO_4) and extracted with chloroform. The chloroform layer is tested for steroids.

Tests for Steroids:

Salkowski Test: Few drops of concentrated H_2SO_4 are added to the plant extract, shaken, and on standing; if the lower layer turns red in color, then it indicates the presence of steroids.

Liebermann-Bur Chard's Test: To the chloroform layer of extract, few drops of acetic anhydride are added from the sides of the test tube if a reddish-brown ring is observed at the junction of the two layers indicates the presence of steroids.

Test for Triterpenoidal Saponins: The extract is hydrolyzed with H_2SO_4 and extracted with chloroform. The chloroform layer is tested for triterpenoids.

Tests for Tri-terpenoids:

Salkowski Test: Few drops of conc. H_2SO_4 is added to the plant extract shaken and on standing, if lower part turns golden yellow in color, indicates the presence of triterpenoids.

Liebermann-Burchard's Test: To the chloroform layer of extract, few drops of acetic anhydride is added from the sides of test tube if a reddish-brown ring is observed at the junction of the two layers indicates the presence of triterpenoids.

Iscugajiu Test: Excess of acetyl chloride & a pinch of zinc chloride are added to the solution and kept aside for reaction to subside and warmed on water bath if Poison red color is produced, indicates the presence of tri-terpenoids.

Brickorn & Brinar Test: To the solution, few drops of chlorosulfonic acid in glacial acetic acid (7:3) are added; if red color is observed, the presence of triterpenoids.

Tests for Saponins:

Foam Test: Small amount of extract is shaken with a little quantity of water, then if the foam is produced and persists for 10 min. It confirms the presence of saponins.

Tests for Alkaloids:

Mayer's Test: Mix the acid layer of the extract with potassium mercuric iodide solution; creamy white precipitate indicates the presence of alkaloids.

Dragendroff's Test: The acid layer of the extract with few drops of Dragendroff's reagent if reddish-brown precipitate indicates the presence of alkaloids.

Wagner's Test: The acid layer of the extract, when mixed with few drops of Wagner's reagent (solution of iodide in potassium iodide) if it gives brown to red precipitate, indicates the presence of alkaloids.

Hager's Test: The acid layer of the extract, when mixed with few drops of Hager's reagent (saturated solution of picric acid) if it gives yellow colored precipitate, indicates the presence of alkaloids.

Tests for Carbohydrates:

Fehling's Test: The extract, when heated with Fehling's A & B solutions, if it gives an orange, red precipitate indicates the presence of reducing sugar.

Molisch's Test: The extract is treated with Molisch's reagent and conc. H_2SO_4 along the sides of the test tube, if a reddish violet ring is observed, indicate the presence of carbohydrates.

Benedicts Test: The extract is heated with Benedict's reagent; if brown precipitate is observed indicates the presence of sugar.

Barfoed's Test: The extract on heating with Barford's reagent on a boiling water bath for a few minutes, a reddish precipitate is observed indicates the presence of carbohydrates.

Tests for Flavonoids:

Shinoda Test: The alcoholic solution with a few fragments of magnesium ribbon and conc. HCl produces a magenta color after a few minutes indicates the presence of flavonoids.

Ferric Chloride Test: Alcoholic solution of bark extract react with freshly prepared $FeCl_3$ if it gives blackfish green color indicates the presence of flavonoids.

Lead Acetate Test: Alcoholic solution of an extract with 10% lead acetate solution gives white precipitate indicates the presence of flavonoids.

Tests for Glycosides:

Anthraquinone Test: Powdered bark extract is extracted with either ammonia or caustic soda. If the aqueous layer shows, pink color indicates the presence of glycosides.

Keller-killiani Test: Chloroform layer of extract and glacial acetic acid with ferric chloride and 0.5 ml of conc. H₂SO₄. If acetic acid layer shows, blue color indicates the presence of cardiac glycosides.

Tests for Phenolic Compounds:

Ferric Chloride Test: Treat the extract with ferric chloride solution if blue color appears, indicates the presence of hydrolyzable tannins, and green color appears indicates the presence of condensed tannins.

Gelatin Test: To the extract, solution add 1% gelatin solution containing 10% NaCl; a precipitate is formed indicates the presence of phenolic compounds.

Test for Chlorogenic Compounds: Treat the solution with aqueous ammonia and expose to air gradually; if green color develops indicates the presence of phenolic compounds, tannins.

Determination of Minimum Inhibitory Concentration (MIC)²¹: The Minimum Inhibitory Concentration (MIC) was determined using serial tube dilution technique. A series of nine test tubes with 1ml of sterilized nutrient broth were serially diluted with 1ml of the sample solution. 10 µl of the inoculum was added to all the serially diluted tubes, positive control and were incubated at 37 °C for 18 h. Two test tubes, one with sterilized media and the other with inoculated media, were used as negative and positive controls, respectively. The dilution of the test tube without growth indicates the MIC of the extract on the given organism. The results were shown in **Table 2**.

Antimicrobial Bioassay by Agar Well Diffusion Method:²² The antimicrobial potential of hydro alcoholic bark extract of *Pongamia pinnata* was evaluated according to their zone of inhibition against various pathogens (*Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*), and the results

were compared with the activity of standard. Muller Hinton agar plates were swabbed with 24 h old broth culture of respective bacteria, and fungi wells were made in each of these plates using a sterile cork borer. The stock solution of plant extract was prepared at concentrations 100, 500, 1000 µg/ml using DMSO. About 100 µl (25mg/ml) of extract dilution was allowed to diffuse into the punched agar medium at room temperature for 2 h. The plates were then incubated in the upright position at 37 °C for 24 h. Wells containing DMSO served as negative control while standard antimicrobials such as ciprofloxacin, fluconazole were used as positive controls. After incubation, the diameters of the growth inhibition zones were measured and tabulated in **Table 3**.

RESULTS AND DISCUSSION: Phytochemical screening of the hydro-alcoholic bark extract of *Pongamia pinnata* revealed the presence of saponins, flavonoids, phenolic compounds, and carbohydrates as major compounds while glycosides and steroids were in minor quantity. The hydroalcoholic bark extract of *Pongamia pinnata* was assessed for their antimicrobial sensitivity and activity with the organisms *Bacillus subtilis*, *Escherichia coli*, and *Aspergillus niger*. The result of phytochemical screening and antimicrobial activity were summarized in **Table 1**, **Table 2**, and **Table 3**, respectively. The MIC of the extract against all the tested organisms was 100 µg/ml, which was consistent and low compared to work done by Anwarul-Hassan Gilani, 2012²³.

The zone of inhibition produced with *Bacillus*, *Escherichia*, and *Aspergillus* range from 18-26 mm, which was significant compared to the zone produced by 0.05% ciprofloxacin (28 mm) and 0.05% fluconazole (32 mm). Hydro-alcoholic bark extract exhibited significant antimicrobial activity on all the tested strains. The results of the antimicrobial assessment were consistent with the previous work done with aqueous methanolic and ethanolic bark extract of *Pongamia*. The previous studies also reveal that the extracts of *Pongamia pinnata* have shown a synergistic effect with the antibiotics like ampicillin, meropenem, cefotaxime, cefazolin and cefuroxime and also reduced the MIC of the respective antibiotics toward the pathogenic bacteria²⁴. The presence of flavonoids, phenolic compounds, and saponins in the plant, which retard

microorganisms' growth by inhibiting the nucleic acid replication, interfere with cytoplasmic membrane function and energy metabolism, might be responsible for the effects observed.

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING OF HYDRO-ALCOHOLIC BARK EXTRACT OF PONGAMIA PINNATA

Tests	Phytoconstituent	Analysis of report
Salkowski Test	Steroids	++
Libermann Burchard's	Steroids	+++
Iscugajiu Test	Triterpenoids	-----
Foam Test	Saponins	+++++++
Dragendroff's Test	Alkaloids	-----
Mayer's Test	Alkaloids	+
Molisch's Test	Carbohydrates	+++++
Benedicts Test	Carbohydrates	+++++
Shinoda Test	Flavonoids	+++
Ferric Chloride Test	Flavonoids	++++
Keller-Killiani Test	Cardiac Glycosides	-----
Antraquinone Test	Glycosides	++
Ferric Chloride Test	Phenolic Compounds	+++++
Gelatin Test	Phenolic Compounds	+++

Note: +++ indicates Miniscule Amount; ++++++ indicates Abundant Amount of, ----- indicates absence of phytochemical in the Extract

TABLE 2: RESULTS OF MIC STUDY OF HYDRO ALCOHOLIC BARK EXTRACT OF PONGAMIA PINNATA

Antimicrobial activity	Minimum Inhibitory Concentration (MIC) in µg/mL		
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Aspergillus niger</i>
<i>Pongamia pinnata</i> bark extract	100	100	100
Ciprofloxacin	50	50	-
Fluconazole	-	-	50

TABLE 3: RESULT OF ANTIMICROBIAL ACTIVITY OF HYDRO ALCOHOLIC BARK EXTRACT OF PONGAMIA PINNATA

Antimicrobial activity	Zone of Inhibition in millimeters (mm)		
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Aspergillus niger</i>
100	18	21	18
500	21	24	24
1000	24	26	26
0.05%	28	28	-
Ciprofloxacin	-	-	-
0.05% Fluconazole	-	-	32

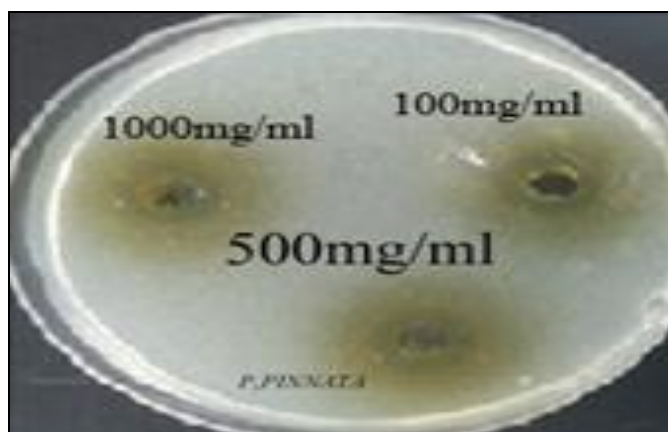


FIG. 1: ANTI BACTERIAL ACTIVITY OF PONGAMIA PINNATA PLANT-BARK EXTRACT ON GRAM POSITIVE AEROBIC BACILLUS SUBTILIS

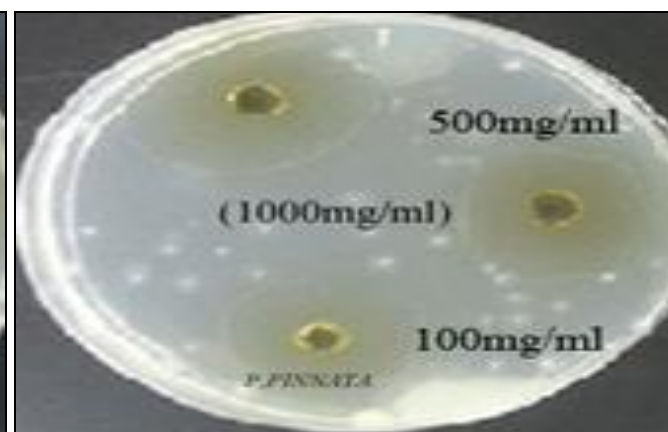


FIG. 2: ANTI BACTERIAL ACTIVITY OF PONGAMIA PINNATA PLANT-BARK EXTRACT ON GRAM NEGATIVE AEROBIC ESCHERICHIA COLI

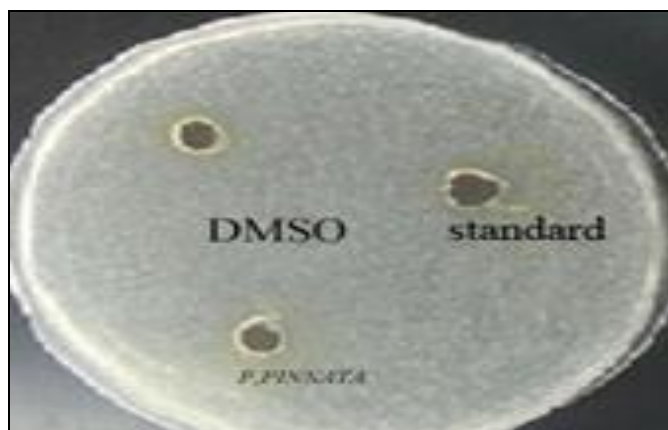


FIG. 3: ANTI BACTERIAL ACTIVITY OF PONGAMIA PINNATA PLANT-BARK EXTRACT ON BACTERIA USING STANDARD

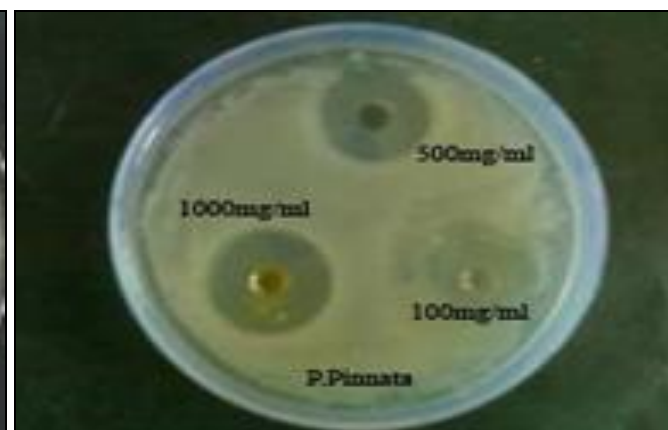


FIG. 4: ANTI FUNGAL ACTIVITY OF PONGAMIA PINNATA PLANT-BARK EXTRACT ON ASPERGILLUS NIGER

CONCLUSION: The present study and the review reveal that the flavonoids (flavones, isoflavone, and chromone) might be responsible for the therapeutic effects previously reported and the antimicrobial action. A future scientific study about isolation and formulation of the same as an alternative for antimicrobial therapy is needed and is under process.

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CONFLICTS OF INTEREST: Authors declare no conflict of interest.

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