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QUALITATIVE PHYTOCHEMICAL SCREENING LEAF, STEM BARK EXTRACTS OF *DIOSPYROS CHLOROXYLON ROXB.*

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ABSTRACT

Diospyros is the most important genus of Ebenaceae family with more than 500 species. These are known for their medicinal use for centuries in traditional systems of India, china and Africa. In addition to their medicinal value, the species have a lot of economic importance. Out of the 60 Indian *Diospyros*, eleven species are distributed in Andhra Pradesh State. In view of the enormous scientific attention being paid to *Diospyros* species, the present study reports the qualitative phytochemical screening of *Diospyros chloroxylon* on which very little research studies have been made from Andhra Pradesh state. The present study records the presence of secondary metabolites in the leaf, stem and bark extracts in *Diospyros chloroxylon*. Methanolic leaf and bark extracts tested positive for the highest number of phytocompounds, when compared to other solvents viz., aqueous and ethyl acetate. Fixed oils and fats were absent in the three solvent extracts of leaf, stem and bark of *Diospyros chloroxylon*. The distributions of fourteen tested compounds vary significantly based on plant part (leaf, stem and bark) and solvent type (ethyl acetate, methanol and water). Further, identification of individual compounds in each group is required to understand their importance in different biological activities.

KEYWORDS: Phytochemicals, Solvents extracts, secondary metabolites biological activities, Synthetic compounds.

INTRODUCTION

Plants are to be considered as indispensable source of medicinal preparations both preventive and curative.[3] Plants synthesize an array of secondary metabolites and identification and characterization of such chemicals will find their potential use in modern drug development.[13] Plant based crude drugs are used throughout the world against various infections and diseases.[9] Majority of the plant based natural drugs are without side effects and mostly preferred despite of commercially available synthetic alternatives in the market. Hence, there is renewed interest today in medicinal herbs which provide many natural compounds. The medicinal importance of a plant depends on its chemical profile by the presence of compounds such as alkaloids, glycosides, resins, volatile oils, gums, tannins etc.,[6] and phytochemical screening help in understanding the actual value of folklore remedies of plants.[2]

Globally many plants species are yet to be investigated phytochemically and pharmacologically.[1] Out of the total 5,00,000 plant species, roughly 2 % have been phytochemically investigated. Therefore, there is an urgent need to carry out phytochemical analysis of plant species to discover many phytochemicals of future potential.[5]

Diospyros is the principal genus of family Ebeanceae with more than 500 species. Of these nearly 60 species occure in India out of which 11 species found in Andhra Pradesh state.[8,10] The species of *Diospyros* are with multiple uses besides possessing a rich reserve of pharmacologically important chemicals that accelerate the pace of drug discovery.[11]

There are very few research studies on *Diospyros* species from Andhra Pradesh state. Hence the present study is aimed at qualitative phytochemical screening of *Diospyros chloroxylon* leaf, stem and bark extracts.

MATERIALS AND METHODS

The stem, bark and leaf of *Diospyros chloroxylon* are collected from its natural habitat (i.e., Kondapalli Reserve forest) in Krishna district (Andhra Pradesh)

India. The plant was identified by its Botanical name at the Deccan Regional Centre Botanical Survey of India, Hyderabad. The voucher specimen number of the plant is BSI/DRC/2019-20/Tech./173. The herbarium specimens were deposited in the department of Environmental Sciences, Acharya Nagarjuna University, Guntur (A.P).

Phyto-chemical screening Methods

All the chemicals that were used were of laboratory reagent grade and were purchased from Merck and Fisher scientific company, Mumbai. The Borosil glassware was used in all the experiments. The leaf, stem and bark extracts of *Diospyros chloroxylon* were used for phyto-chemical analysis by using soxhelt extraction method with Ethyl acetate, Methanol and Water as solvents.

Preparation of the extract

Leaves, stem and bark (500g each) of *Diospyros chloroxylon* were used for extraction. The air dried material was made into a fine powder and taken into a conical flask. Ethyl acetate, methanol and water were used for extraction. The extraction was performed by soxhlation and the extraction was carried out until the extract becomes colour less. The pooled extract was distilled under reduced pressure into syrup and evaporated in a porcelain basin over a water bath to give a sticky residue. This was kept in a desiccator for a few days to get the dry extract (Yield-500g).

Preparation of the reagents

Bromocresol green solution was prepared by heating 69.8 mg bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until completely dissolved and the solution was diluted to 1000 ml with distilled water. ***Phosphate buffer solution (pH 4.7)*** was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na2HPO4 in 1 L distilled water) to 4.7 with 0.2 M citric acid (42.02 g citric acid in 1 L distilled water).

Folin- Ciocalteu's (FC) reagent**:** 10ml of Folin-Ciocalteu's solution was dissolved in 90ml of double distilled water.

Iron (III) chloride solution**:** 500mg of ferric chloride was weighed and dissolved in 100ml of distilled water.

Potassium hexacyanoferrate (III) **solution:** 500mg of potassium hexacyanoferrate was weighed and dissolved in 100ml of distilled water.

Qualitative phytochemical screening tests

Tests for Alkaloids: The extracts were separately treated with few drops of dilute hydrochloric acid and filtered. The filtrate was tested for the presence of alkaloids.

Hagner's test**:** Extract treated with Hagner's reagent (Picric acid solution) - yellow precipitate indicates alkaloids.

Mayer's test**:** Extract treated with Mayer's reagent (Potassium mercuric iodide solution) - cream precipitate indicates alkaloids.

Dragendroff's test**:** Extract treated with Dragendroffs reagent (Potassium bismuth iodide solution) - orange precipitate indicates alkaloids.

Wagner's test**:** Extract treated with Wagner's reagent (Iodine-potassium solution) - reddish brown precipitate indicates alkaloids.

Tests for Carbohydrates: Small quantities of extracts were separately dissolved with 5 ml of distilled water and filtered. The filtrate was subjected to following tests.

Molisch's test**:** Extracts treated with Molisch reagent (alpha napthol in 95% ethanol) and few drops of concentrated hydrochloric acid at the sides of the test tube gives violet ring at the junction.

Fehling's test**:** Extract treated with Fehling reagent (Fehling's reagent A) Copper sulphate in water and Fehling's reagent B – (Sodium potassium tartarate) gives red colour.

Barfoed's test**:** Extract treated with Barfoed reagent (Copper acetate in water and glacial acetate) gives red colour.

Benedict's test**:** Extract treated with Benedict reagent (Copper sulphate, sodium citrate and sodium carbonate in water) gives red colour.

Test for Glycosides: The extracts were separately hydrolyzed with dilute hydrochloric acid for few hours in a water bath and then subjected to following tests.

Libermann Burchard's test**:** Extract was treated with chlorform in a dry test tube along with a few drops of glacial acetic acid and a few drops of concentrated sulphuric acid at the sides of the test tube. A red color at the junction of two layers and the upper green colour indicates glycosides.

Legal test: Extract treated with Disodium nitroprusside in pyridine and sodium hydroxide gives red colour.

Bomtrager's test**:** Extract treated with diluted sulphuric acid and solvent ether on shaking produce the organic layer which on addition of ammonium solution produce organic layer becomes pink to red.

Test for Phytosterols: The extracts were refluxed with alcoholic potassium hydroxide till complete saponification takes place. The saponification mixture was diluted with distilled water and ether. Evaporated the ethereal extract and subjected the residue to the tests below.

***Liberman Burchard's test*:** Extract was treated with chlorform in a dry test tube and few drops of glacial acetic acid and few drops of concentrated sulphuric acid at the side's of the test tube. A red color at the junction of two layers and the upper layer shows green color.

***Salkowski test*:** Extract was treated with equal volumes of chloroform and sulphuric acid, Red or violet color, indicates phytosterols.

Test for Saponins: (a) *Foam test*: l ml of extracts were diluted separately with distilled water (20 ml) and thoroughly shaken in a graduated cylinder for 15 min. A layer of one centimeter foam indicates the presence of Saponins.

***(b) Haemolysis test*:** 2ml of 1.8% sodium chloride solution was taken in two test tubes. To one test tube 2ml of distilled water was added and to the other 2ml of 1% filtrate. Blood is obtained by pricking the thumb and 5 drops of blood were added to each tube, the contents were gently mixed and observed under microscope. Haemolysis that occurs, indicates the presence of Saponins.

Test for Tannins: Small quantities of extracts were diluted separately with distilled water and subjected to following tests.

***Ferric chloride test*:** Extract treated with ferric chloride solution gives blue colour.

***Gelatin test*:** Extract treated with gelatin solution gives white precipitate.

***Lead acetate test*:** Extract treated with lead acetate solution gives yellow precipitate.

Test for Proteins and amino acids
Small quantities of extracts were separately diluted with a few ml of distilled water and then subjected to tests viz., millions test, biuret test, ninhydrin and sodium bicarbonate.

***Millions test*:** Extract treated with Million's reagent (Mercuric nitrate in nitric acid) gives red color.

***Biuret test*:** Extract treated with sodium hydroxide and copper sulphate solution added drop wise and mixed that gives violet color.

***Ninhydrin test*:** Extract treated with Ninhydrin reagent (Ninhydrin with alpha amino acid) and a mmonium and heated it gives, violet color.

***Sodium bicarbonate test*:** Extract was treated with sodium bicarbonate solution to give brisk effervesces.

Test for Flavonoids: (i) *Ferric chloride test*: To the alcoholic extract a few drops of neutral ferric chloride solution was added to give blackish red color. **(ii) *Lead acetate test*:** To the alcoholic extract lead acetate solution was added that gives yellow precipitate. **(iii) *Shinda's test*:** To the alcoholic extract a few fragments of magnesium ribbon and concentrated hydrochloric acid were added along the side of test tubes that gives magenta color. (i**v) *Zinc-hydrochloric acid test*:** To the alcoholic extract, a pinch of zinc dust and concentrated hydrochloric acid along the side of test tubes were added to produce Magenta color. **(v) *NaoH test*:** Extract samples were reduced in water bath. The residue was treated with dilute NaoH along with dilute HCl. The solubility and colouration was noted. A yellow coloured precipitation which turns color less with the addition of dilute HCl confirms the flavonoids.

Borntgers test for Anthraquinones: 0.5 g of plant extract was taken into a dry test tube containing chloroform, shaken for 5 min and filtered. Equal volume of 10% ammonia solution was added. Pink violet or red colour in the ammonical layer indicates positive results for anthraquinones.

Test for Terpenoids: A volume of 5 ml of the plant extract was mixed in 2 ml of chloroform and concentrated H_2S0_4 was added to form a layer. A reddish brown coloration of the interface was formed indicating the presence of terpenoids.

Keller-Killani Test for Cardiac Glycosides: Extract was dissolved in 2ml of chloroform and sulphuric acid. Formation of brown ring at interphase confirms the presence of cardiac glycosides.

Test for Phlobatannins: The extract (0.5 g) was dissolved in distilled water and filtered. The filtrate was boiled with 2% HCl. Formation of red precipitate indicated the presence of phlobatannins.

Test for Fixed oils and fats: A drop of concentrated extracts was pressed in between two filter papers and kept undisturbed. Oil strains on the paper indicate the presence of oils and fats.

Test for Phenolic Compounds: (i) *Ferric chloride test*: The extract was treated with ferric chloride solution. Blue color indicates hydrolysable tannins and green color indicate condensed tannins in the extract .**(ii) *Gelatin test*:** 1% gelatin solution containing 10% Nacl was added to the a precipitate formation indicates phenolic compounds test solan.

RESULTS

The leaf, stem and bark extracts of *Diospyros chloroxylon* were prepared with methanol, ethyl acetate and aqueous solvents and the extraction quantities are furnished in table 1. Methanol solvent gave the highest extraction quantity for leaf (25.7%), stem (17.3%) and bark (18.5%) followed by aqueous extract. The

extraction quantity of ethyl acetate solvent was the lowest compared with other solvents.

Qualitative Phytochemical screening for fourteen different compounds was conducted, in the leaf, stem and bark of *Diospyros chloroxylon* with three solvents. The leaf, stem and bark extracts were subjected to qulatitive phytochemical tests to detect alkaloids, amino acids, anthraquinones, carbohydrates, cardiac glycosides, fixed oils & fats, flavonoids, glycosides, phenolic compounds, phytosterols, proteins, saponins, terpenoids and phlobatannins. Amino acids and fixed oils & fats were not detected in the three solvent extracts of leaf, stem and bark (Table 2). Aqueous leaf extracts and methnolic leaf extracts tested positive for the majority of test compounds, where as ethyl acetate leaf extracts tested positive only for cardiac glycosides, glycosides, phytosterols and terpenoids.

Aqueous stem extracts of *Diospyros chloroxylon* showed the presence of majority of tested compounds viz., Anthraquinones, carbohydrates, proteins, saponins, terpenoids and phlobatannins, where as ethyl acetate stem extracts were tested positive for five compounds (anthraquinones, cardiac glycosides, glycosides, proteins and terpenoids). The methanolic stem extracts were tested positive only for flavonoids, phenolic compounds and terpenoids (Table 2).

Methanolic bark extracts revealed the presence of highest number of compounds i.e., anthraquinones, carbohydrates, flavonoids, phenolic compounds, proteins, saponins, terpenoids and phlobatanins. Aqueous extracts of bark tested positive for only anthraquinones, phenolic compounds and saponins only (Table 2). The bark in ethyl acetate solvent tested positive only for anthraquinones, phytosterols and terpenoids.

Alkaloids were tested positive only in aqueous leaf extracts. Anthraquinones were tested positive in three solvent extracts of bark. In case of leaf, methanoilc extracts only revealed the presence of anthraquinones where as stem extracts of aqueous and ethyl acetate solvents tested positive for anthraquinones. Carbohydrates were detected in aqueous leaf, stem and also in methanolic bark extracts. Cardiac glycosides were only present in ethyl acetate extracts of leaf and stem. Flavonoids were found in methanolic leaf and stem only. Glycosides were detected in leaf extracts of three solvents and ethyl acetate stem extracts. Aqueous and methanolic extracts of leaf and bark exhibited phenolic compounds where as stem methanolic extracts only showed the phenolic compounds. Phytosterols were tested positive in leaf extracts but absent in stem extracts. The proteins were not detected in leaf extracts, while they were tested positive with both stem aqueous and ethyl acetate extracts. Terpenoids were found in leaf and stem extracts, while aqueous extract of bark did not contain them. The phlobatanins were present in methanolic extract of leaf and bark and in the aqueous extracts of stem only. Saponins were found in leaf and bark.

Table 1: Extraction quantity of *Diospyros chloroxylon* leaf, stem and bark.

S No	Name of the plant part	Solvent Name	Extract obtained in %
1	***Diospyros chloroxylon* Leaf**	Ethyl acetate	2.6% w/w
2		Methanol	25.7% w/w
3		Aqueous	19.6% w/w
4	***Diospyros chloroxylon* Stem**	Ethyl acetate	2.1% w/w
5		Methanol	17.3% w/w
6		Aqueous	20.8% w/w
7	***Diospyros chloroxylon* Bark**	Ethyl acetate	1.9% w/w
8		Methanol	18.5% w/w
9		Aqueous	16.2% w/w

Table 2: Preliminary phytochemical analysis of *Diospyros chloroxylon* leaf, stem and bark extracts.

S No	Phyto constituent	Test results observed for extract								
		Ethyl acetate			Methanol			Water		
		L	S	B	L	S	B	L	S	B
1	Alkaloids	-	-	-	-	-	-	+	-	-
2	Amino acids	-	-	-	-	-	-	-	-	-
3	Anthraquinones	-	+	+	+	-	+	+	+	+
4	Carbohydrates	-	-	-	-	-	+	+	+	-
5	Cardiac Glycoside	+	+	-	+	-	-	+	-	-
6	Fixed oils and fats	-	-	-	-	-	-	-	-	-
7	Flavonoids	-	-	-	+	+	+	+	-	-
8	Glycosides	+	+	-	+	-	-	+	-	-
9	Phenolic Compounds	-	-	-	+	+	+	+	-	+
10	Phytosterols	+	-	+	+	-	-	+	-	-

11	Proteins	-	+	+	-	-	+	-	+	-
12	Saponins	-	-	-	+	-	+	+	+	+
13	Terpenoids	+	+	+	+	+	+	+	+	-
14	Phlobatanins	-	-	-	+	-	+	-	+	+

- Indicates Negative test L – Leaf S- Stem B- Bark + Indicates Positive Test

DISCUSSION

In Ayurveda, Yunani and other Indian traditional systems twelve (12) *Diospyros* species have been extensively used to cure rheumatism, (*Diospyros candollena*), diarrhoea and dysentery (*Diospyros exsculpta*), cholera and menorrhagia (*Diospyros malabarica*), skin and urinary diseases (*Diospyros melanoxylon*), tuberculosis, jaundice and pneumonia (*Diospyros Montana*), urinary infections and snake bite (*Diospyros perigrina*) and cholera, bronchitis, cough, syphilis, tumors, fistula (*Diospyros tomentosa*). Every species is specific in producing a characteristic mixture of secondary metabolites.[4]

Rauf *et al*.[11] commented that the *Diospyros* is a versatile genus with immense utility. Out of 500 species only 100 species were phytochemically studied and the rest need phytochemical screening.[12] Therefore, the literature survey on Indian *Diospyros* species revealed that very few scientific investigations were done on *Diospyros chloroxylon Roxb.* Moreover, Mallavadhani *et al*.[7] reported that the quantity of phytocompounds such as betulinic acid present in *Diospyros tomentosa* was positively correlated to the altitude at which the species grow. The phytocompounds from *Diospyros* species were responsible for various biological activities. In the back drop of the colossal importance attached to the *Diospyros* species, the present phytochemical analysis of locally available (Kodapalli Reserve Forest-Krishna District Andhra Pradesh) *Diospyros chloroxylon* plant becomes very pertinent study.

The secondary metabolites tested positive in leaf, stem and bark of *Diospyros chloroxylon* are of great significance since they involve in many active biological activities that collectively enhance the value of *Diospyros chloroxylon* as an important plant to be grouped with the other *Diospyros* species of established medicinal importance. The methanol extract and aqueous extracts were proved better over ethyl acetate solvent since majority of chemical compounds were found in them.

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