

PHYTOCHEMICAL SCREENING AND HPLC ANALYSIS OF FLAVONOID AND ANTHRAQUINONE GLYCOSIDE IN ZANTHOXYLUM ARMATUM FRUIT

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ABSTRACT

Objective: The present paper deals with the phytochemical screening of *Zanthoxylum armatum* fruits, an important medicinal plant of J&K region. This study involves the preliminary screening. Further HPLC flavonoid and glycoside profile of the methanolic extract had been studied.

Methods: The retention behavior of flavonoid and glycoside isolated in methanolic extract of *Zanthoxylum armatum* fruits in a reversed-phase (RP) high performance liquid chromatography (HPLC) system has been studied on a C18-RP column using acetonitrile-water and methanol-water mixtures as mobile phase.

Results: The preliminary screening test results in the detection of bioactive principles and the retention time is identified in the HPLC chromatogram, as flavonoid ($R_f=2.8$) and anthraquinone ($R_f=2.2$).

Conclusion: The generated data has provided the basis for its wide uses as the therapeutic in the traditional and folk medicine.

Keywords: Flavonoid, Glycoside, HPLC, Phytochemical, *Zanthoxylum armatum*

INTRODUCTION

The flavonoids and glycoside belong to one of the most bioactive compounds which naturally exist in the plant kingdom. Different naturally occurring flavonoids have been described and subcategorized into flavones, flavans, flavanones, isoflavonoids, chalcones, aurones and anthocyanidines [11, 12]. These flavonoids have remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types, protection against allergies, antiviral, anti-malarial, antioxidant, anti-inflammatory and anti-carcinogenic properties [11, 12].

Anthraquinones are a class of natural compounds that consists of several hundreds of compounds that differ in the nature and positions of substituent groups [13]. This class of compounds contains derivatives that consist of the basic structure of 9, 10 anthraquinone [14]. Anthraquinones are widely applied in medicine, food and the dye industry. In the pharmaceutical industry, the natural and synthetic derivatives of 9, 10 anthraquinone are beneficial to mammals and humans as they can display antibacterial, antitrypanosomal and antineoplastic activities [15], [16].

Zanthoxylum armatum (TUMBURU) Tumburu consists of dried fruit of *Zanthoxylum armatum* DC. Syn. *Z. alatum* Roxb. *Zanthoxylum armatum* DC. belongs to family Rutaceae. Commonly known as 'timur' or 'Nepali Dhania'. Fruits of *Z. armatum* are a well-known ayurvedic medicine. The fruit and seeds are employed as aromatic and tonic, in fever, dyspepsia and expelling round worms [3]. Fruit, branches and thorn are used to cure toothache and other diseases of teeth. It is considered good for Asthma [1]. Bark powder mixed with honey gives relief against gum bleeding [2]

The plant has been reported to possess antioxidant[4], antinociceptive[4], anti-inflammatory [4,5], natural piscicide[6], antimicrobial[7], antihelmintic[7], hepatoprotective [8], antiproliferative[9], and antifungal activities[10].

The medicinal importance of a plant is due to the presence of some special substances like flavonoids, glycoside, resins, phenolic compounds and Tannins etc. Considering all these facts, presents investigation is designed to find out phytochemical investigation of *Zanthoxylum armatum* a plant which evokes various therapeutic effects and HPLC analysis of isolated compounds.

MATERIALS AND METHODS

Plant Materials

Fresh Fruits of *Zanthoxylum armatum* were collected from, Poonch district, J&K region, India. The flowering period is from March to April. The specimen was authenticated by Botanist Dr. S.N. Sharma, Department of Taxonomy, I.I.I.M, Jammu, India. A voucher specimen CDR No- 1832 was deposited in the Department for future records.

Extraction and Isolation

The shade dried coarse powder of the fruits of *Zanthoxylum armatum* (600g) were extracted with methanol by cold maceration process. The extract was drained, filtered using muslin cloth and concentrated under reduced pressure using rotary film evaporator. The extraction process was repeated three times more under similar conditions. The combined extract was condensed and used for preliminary screening of phytochemicals.

Fractions were eluted in 40% ethylacetate in dichloromethane. Yielded a crude compound (0.0056g), which was recrystallized by repeatedly washing with warm methanol and acetone mixture, to obtain pure pale yellow amorphous powder (0.0023g). m.p 310-315°C, $R_f=0.64$ and shinoda test shows positive result for flavonoids.

Fractions were eluted in 30% methanol in ethylacetate ie. EA: MeOH (70:30). Yielded a crude compound (0.0080g), which was recrystallized by repeatedly washing with warm methanol and acetone mixture, to obtain pure pale yellow amorphous powder (0.0058g). m.p 271-275°C, $R_f=0.64$ and Borntrager's test shows positive result for glycosides

Phytochemical analysis of Methanolic Crude Extract

Methanolic extract was tested for the presence of active principles such as alkaloids, glycosides, carbohydrates, phenolic compounds, flavonoids, tannins and fixed oils and fats. Following standard procedures are followed [17,19,20]

Test for Alkaloids

Dragendorff's Test: To 1 ml of the extract/test solution, add 1 ml of dragendorff's reagent (Potassium Bismuth iodide solution). An orange-red precipitate indicates the presence of alkaloids. **Mayer's Test:** To 1 ml of the extract, add 1 ml of mayer's reagent (Potassium mercuric iodide solution). Formation of whitish yellow or cream coloured precipitate indicates the presence of alkaloids.

Test for Saponins

Frothing test / Foam test Take small quantity of extract separately and add 20 ml of distilled water and shake in a graduated cylinder for 15 minutes lengthwise. A 1cm layer of foam indicates the presence of saponins

Test for Glycosides

Borntrager's Test: Add a 1ml of dilute Sulphuric acid to 1ml of the extract solution. Boil, filter and shake with equal volume of dichloromethane or chloroform. Separate the lower layer of dichloromethane or chloroform and shake it with half of its volume of dilute ammonia. The formation of rose pink to red colour of the ammonical layer shows the presence of anthraquinone glycosides. In some cases the anthraquinones may not answer for bontrager's test due to its reduced form, in that time ferric chloride is used, this test may called modified bontrager's test.

Test for Carbohydrates and Sugars

Molisch's Test: To 2ml of the extract, add 1ml of α -naphthol solution, add concentrated sulphuric acid through the side of the test tube. Purple or reddish violet colour at the junction of the two liquids reveals the presence of Carbohydrates.

Fehling's Test: Add 25ml of diluted sulphuric acid (H_2SO_4) to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide to pH-7 and 5ml of Fehling solution brick red precipitate indicates the presence of sugar (reducing sugar)

Test for Tannins and Phenolic Compounds

Braemer's test: To 1ml of the extract, add ferric chloride solution, formation of a dark blue or greenish black colour product shows the presence of tannins.

Test for Flavonoids

Preparation of test solution

Shinoda's Test: The alcoholic extract of powder treated with magnesium foil an concentrated HCl give intense cherry red colour indicates the presence of flavonones or orange red colour indicates the presence of flavonols.

Test for Steroids

Libermann-Burchard Test: 1gm of the test substance was dissolved in a few drops of chloroform, 3ml of acetic anhydride, 3ml of glacial acetic acid were added, warmed and cooled under the tap water and drops of concentrated sulphuric acid were added along the sides of the test tube. Appearance of bluish-green colour shows the presence of sterols. Salkowski Test: Dissolve the extract in chloroform and add equal volume of conc. H_2SO_4 . Formation of bluish red to cherry colour in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

Test for Proteins and Amino Acids

Biuret Test: Add 1ml of 40% sodium hydroxide solution and 2 drops of 1% $CuSO_4$ solution till a blue colour is produced, then add to the 1ml of the extract. Formation of pinkish or purple violet colour indicates the presence of proteins.

Ninhydrin Test: Add two drops of freshly prepared 0.2% ninhydrin reagent (0.1% solution in n-butanol) to the small quantity of extract solution and heat. Development of blue colour reveals the presence of proteins, peptides or amino acids.

Test for Triterpenoids

Noller's Test: Dissolve two or three granules or tin metal in 2ml thionyl chloride solution. Then add 1ml of the extract into test tube and warm, the formation of pink colour indicates the presence of triterpenoids.

Test for Fixed Oils and Fats

Saponification test: To 1ml of the extract, add few drops of 0.5 N alcoholic Potassium hydroxide along with a drop of phenolphthalein.

Heat the mixture on a water bath for 1-2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Test for Gums and Mucilage

Add about 10ml of aqueous extract slowly to 25ml of absolute alcohol with constant stirring. Filter the precipitate and dry in air. Examine the precipitate for its swelling properties and for the presence of carbohydrate.

HPLC study of Flavonoid and Glycoside profile of methanolic extract of *Zanthoxylum armatum* fruits

The HPLC analysis was performed using a LC-solution, Shimadzu™, MAO 1527,USA with LC-UV-100 UV detector, A CAPCELL(C-18)Column RP (18.5 μ m, 250 \times 4.0 mm),type MG 5-20 μ m, number AKAD/05245 was used for the chromatographic separations. The mobile phase consisted of solvent mixtures [Methanol: Water (80:20)] ratio and [ACN: Water] (80:20) ratio was performed using isocratic elution (0-12min) with a flow rate of 1.0ml/min and a column temperature of 30°C. The injection volume was 20 μ l, and UV detection was effected at 276 nm and 273 nm. HPLC grade solvents were obtained from S.D Fine Chemical Ltd.Mumbai.

Determination of HPLC retention times

Stock solutions of the isolated compounds were prepared in HPLC grade methanol at a concentration of 100 μ gml⁻¹ and stored in a refrigerator until use. All samples were stored at 4°C and were filtered through a 0.45 μ m filter before undertaking HPLC analysis

A 20 μ l portion of these working standards were injected to HPLC system with a 10 μ l loop at ambient temperature and a detector wavelength of 276 nm is selected for the detection of Flavonoid(compound-1) and a detector wavelength of 273 nm is selected for the detection of Glycoside(compound-2).The separated compound-1and compound-2 were initially identified by direct comparison of their retention times as mentioned in literature[18]. The results of HPLC analysis of flavonoid and glycoside were given in Table: 2

RESULTS AND DISCUSSION

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as Alkaloids, Glycosides, Tannins and Phenolic compounds, Flavonoids, Steroids etc. The successive extraction of *Zanthoxylum armatum* fruits in methanolic solvent system revealed the presence of Glycosides, Tannins and Phenolic compounds, Flavonoids etc as shown in Table: 1.

Thus, the preliminary screening test may be useful in the detection of bioactive principles and subsequently may lead to the drug discovery and development.

HPLC analysis of methanolic extract of *Zanthoxylum armatum* fruits is done. The HPLC chromatograph will help as standard chromatogram in future studies, comparing the retention time of isolated compounds with given literatures. The good separation of the peaks which could be identified in the chromatogram, as flavonoid ($R_t=2.8$) for compound I at λ_{max} 277 nm and anthraquinone ($R_t=2.2$) for compound II at λ_{max} 273 nm as shown in Table: 2

Table 1: Phytochemical Screening of Methanolic extract of *Zanthoxylum armatum* fruits

Phytoconstituents	Methanol Extract
Alkaloids	+
Saponins	-
Glycosides	+
Carbohydrates	+
Tannins & Phenolic compounds	+
Flavonoids	+
Steroids	+
Proteins and Amino acids	-
Triterpenoids	-
Fixed Oils and Fats	+
Gums and Mucilage	-

Note: + indicates present; - indicates absent

Table 2: Retention time data of flavonoid (fig: 1) and anthraquinone glycoside (fig: 2).

Figure	Retention Time (R _i)
1	2.845
2	2.269

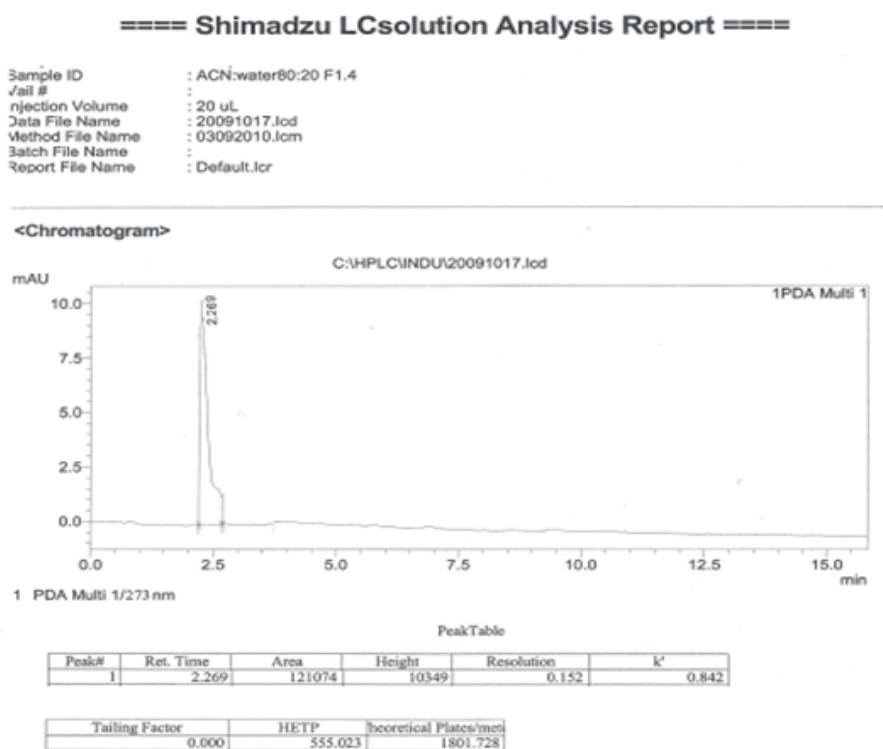


Fig. 1: HPLC Chromatogram of flavonoid

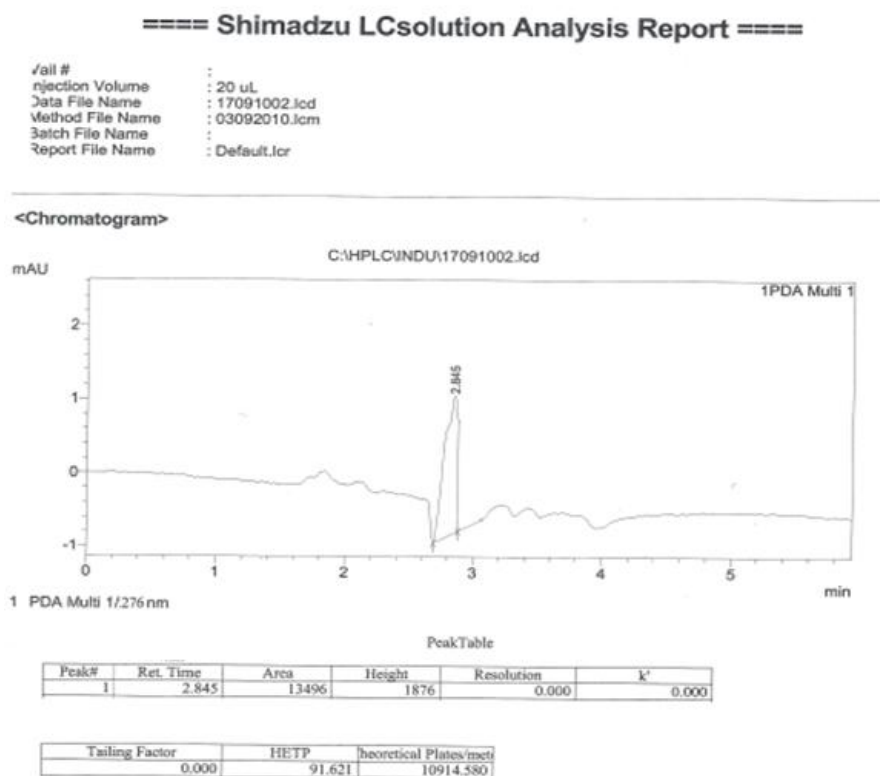


Fig. 2: HPLC Chromatogram of Anthraquinone glycoside

CONCLUSION

In the present study, we have found that the medicinal properties of *Zanthoxylum armatum* fruits may be the presence of above mentioned phytochemicals. The HPLC chromatograph will help as standard chromatogram in future studies, comparing the retention time of isolated compounds with given literatures. Hence these chromatogram can be used as fingerprint for the compound obtained from this plant. The method developed for HPLC

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