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Research Article

QUANTIFICATION OF A PHYTOTOXIN FROM WALNUT SPECIES BY HPTLC METHOD

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ABSTRACT

Objective: Present study deals with the isolation of bioactive component juglone from stem bark extracts of *J. regia* and its quantification by a widely used HPTLC method.

Methods: *Juglans regia* stem bark was powdered and extracted using non polar to polar solvents. The potent molecule juglone was achieved by executing chromatographic technique. Its structure was confirmed by analysis of spectral data. A precise analytical technique (HPTLC) for the quantification was manifested.

Results: Stem bark extracts with solvents chloroform(**A**), ethyl acetate(**B**), acetone(**C**), ethanol(**D**) and methanol(**E**) were analyzed chromatographically to achieve 5-hydroxy-1,4-naphthaquinone (Juglone) as yellow crystalline needles. Quantification of this compound from extracts (**A-E**) was performed. The results of HPTLC experiment reveal that the acetone extract is rich (151.0µg/gm) in this bioactive compound whereas methanol extract contains least amount (29.34µg/gm) of it.

Conclusion: 5- Hydroxy 1, 4- Naphthaquinone (Juglone) had been isolated from the acetone extract of *J. regia* stem bark. This biologically active compound was estimated quantitatively from various bark extracts by HPTLC method. The proposed method was found to be simple, rapid, accurate, precise, economic, reliable and reproducible.

Keywords: *Juglans regia*, Juglone, Acetone extract, HPTLC

INTRODUCTION

Natural products play a very important role in the field of medicine as they offer a vast chemical diversity. Naphthaquinones have a variety of applications in the field of pharmacols, cosmetols and agro ecosystems [1]. Naphthaquinones compared to other natural compounds are very toxic to mosquito larvae. Substituted naphthaquinones and derivatives can be promising larvicidal drugs. Juglone, a naphthaquinone derivative is the important component of family Juglandaceae. It possesses insecticidal, herbicidal and allelochemical properties. Traditionally, it has been used as natural dye, ink and hair dye [2,3]. Juglone exerts its effect by inhibiting certain enzymes needed for metabolic functions. 5-hydroxy-1,4-naphthalenedione (Juglone) occurs naturally in the roots, husks, bark and leaves of Juglandaceae family, particularly *Juglans nigra*. Juglone can be synthesized [4] and isolated from hulls of *J. regia* by sublimation [5] and from *Diospyros lycioides* [6]. It may be formed from glycoside precursor of leaves and also in soil beneath the tree [7]**.** Juglone is involved in pathogenic defense mechanisms and developmental processes [8], thus behaves as a phytotoxin [9].

Bioactivities of juglone and related naphthaquinones are reported. It is used in folk medicine to treat ringworms [5] and found to be potent inhibitor of Herpes Simplex Virus type-1 (HSV-1) [10]. Juglone is an inhibitor of the peptidyl-prolyl isomerase Pin1 [11]. Hyperpigmentation and contact dermatitis is observed [12]. It is a powerful fungicide [9]. Juglone derivatives are potential inhibitors of *Trypanosoma cruzi* (TcTR). Induction of quinine reductase and glutathione transferase in rat tissues by juglone and plumbagin is noted [13]. Its presence is demonstrated in culture of *actinomycete streptoverticillium heroshimense* strain 34. It inhibits the growth of oral cariogenic bacteria (*S. mutans and S. sanguis*) and periodontal pathogens (*P. gingivalis and P. intermedia*) [6]. The activity tested against oral bacteria displays significant results [14]. Aqueous extract exhibits antifungal activity [15]. Isomeric juglones show positive inotropic and chronotropic action on the frog heart indicating involvement of calcium channels. Sedative effect of juglone is studied [16]. It possesses allelochemical properties [1], antifungal [9] and antimicrobial activity. Formerly this compound has been estimated using various methods. A method for its separation from chloroform extract was developed [17]. HPLC was used for quantitative determination of juglone in fresh leaves of *J. regia* [18]. Present study deals with systematic analysis to evaluate therapeutic potential juglone from stem bark extracts of *J. regia,* its quantification by HPTLC and development of simple and common solvent system for it. It is an accurate and reliable technique for quantification of this compound from various crude extracts.

MATERIALS AND METHODS

Isolation of bioactive component

Pulverized bark material (100 g) was extracted with acetone (500 ml) by refluxing for 18 hours. Solvent was recovered under reduced pressure to yield crude mass (9.68%), which (7g) was adsorbed on silica gel (60-120,10 g) and broad fractioned by stirring using non polar to polar solvents. Total eleven broad fractions were collected. The fractions were monitored by TLC. The crude fraction [**4**, toluene: ethyl acetate (3:1)] showed presence of bioactive naphthaquinone along with some unidentified compounds. The impure fraction (**4, 3.8 %**) was dissolved in ether. Ether soluble part was purified by repeated crystallization using methanol as a solvent. The structure was confirmed by analysis of FTIR, UV-Vis, LC-MS, ^{1}HNMR, ^{13}CNMR spectra.

Quantification by HPTLC Method

The isolated compound was assigned to be an authenticated compound, Juglone. The compound was procured from Aldrich Chemical Company Mumbai, Maharashtra, India. This compound was referred as standard for HPTLC experiment. Quantification of this compound was achieved from different extracts of stem bark material of *J. regia* L. The experimental conditions are described as

Chromatographic Experimental Conditions

Stationary phase:	Pre-coated silica gel plates Merck60F254 (10×10cm,0.2mm)
Mobile phase:	Hexane: Ethyl acetate (9:1)
Lamp:	Deuterium
Wavelength:	258 nm
Application mode:	CAMAG Automatic TLC Sampler III
Development mode:	CAMAG Twin Trough Chamber
Scanner:	CAMAG TLC Scanner 3 and CATS software
Experimental conditions:	Temperature 25±2^{0}C, relative humidity 40%

Preparation of Standard Stock Solution and Extracts

A stock solution (1mg/ml) of standard was prepared in methanol and was further diluted with methanol for working standard solution of 0.2mg/ml. Air shade dried, pulverized plant material was extracted for 18 hours with chloroform, ethyl acetate, acetone, ethanol and methanol to capitulate the respective crude extracts (**A-E**).

Calibration curve for standard

The standard solution of juglone (1µg to 5µg per respective spot) was applied in triplicate on TLC plate. Quantitative evaluation of the plate was performed in absorption / reflection mode at 258 nm using a slit width of 6.0 × 0.30 mm, scanning speed 20 mm/s with a computerized CAMAG TLC Scanner-3 integrated with CATS - III software. The plate was developed and scanned as per the chromatographic conditions and the peak areas were recorded.

HPTLC Quantification in Test Samples

The extracts (**A-E**) were used for experiment. Each test sample was diluted (1 mg/ml) with respective solvent; 20µL per spot of these solutions were applied on plate in triplicate. The plates were developed by ascending mode to a distance of 10cm and scanned as per the conditions mentioned above. The content of compound from various extracts was determined by comparing the area of the chromatogram with the calibration curve of working standard. Average content of juglone in different extracts was expressed as µg/g of extract. This quantification method for this bioactive molecule was performed for the first time

RESULTS AND DISCUSSION

Figure-1 and **Figure-2** display calibration curve and HPTLC scan of the standard component. **Figure-3** shows peak areas of extracts and **Figure-4** is for quantification results of extracts.

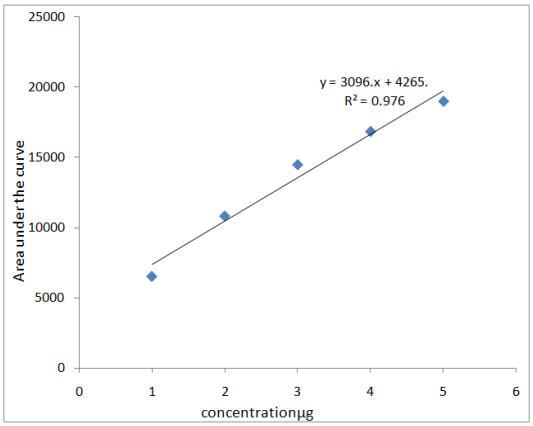

Fig. 1: Calibration Curve for Standard

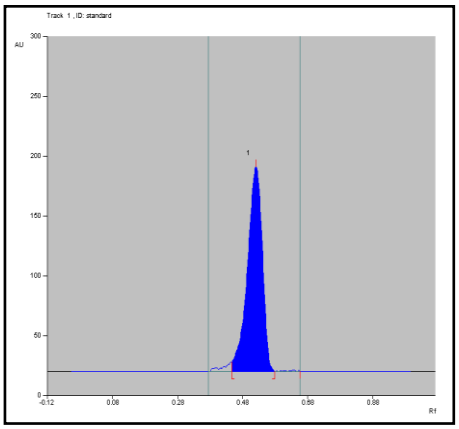

Fig. 2: HPTLC Scan of Standard

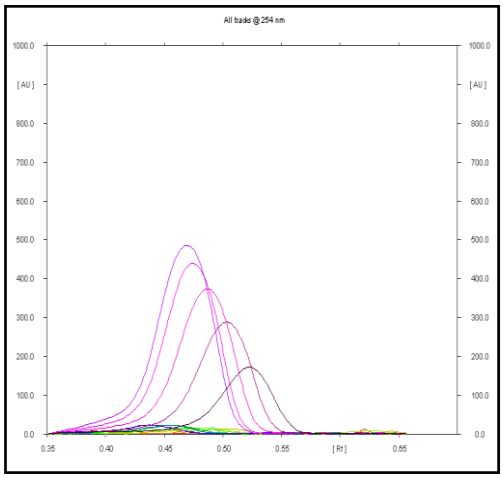

Fig. 3: Peak Areas of Extract

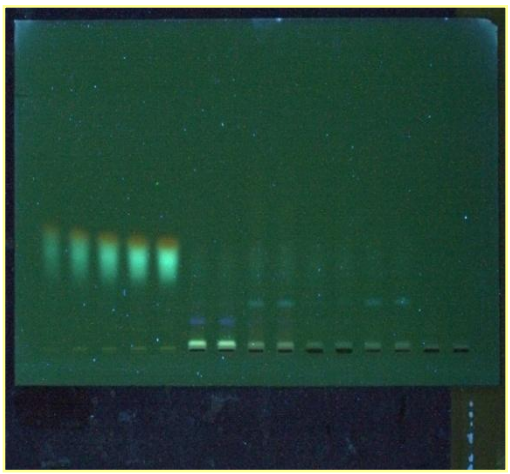

Fig. 4: Quantification in test Samples

Juglone was isolated as yellow crystalline needles. It showed sharp melting nature at 1740C. The mass spectrum of the compound displayed a molecular ion peak at m/z 173 [m-1]+ which suggested the molecular formula to be C10H6O3 . The IR spectrum showed absorption bands at 3444 cm-1 (-O-H stretching), 1717, 1653 cm-1 (α- β unsaturated ketone) 1644cm-1 (double bond), 1575, 1487 cm-1 (aromatic stretching). UV-VIS spectrum assigned λ max at 423 nm. 1H and 13C NMR spectra were compared with those of the authentic sample.

5-hydroxy 1, 4-naphthaquinone

The existence of esteemed compound was detected from different extracts of stem bark material by HPTLC for the first time. Various compositions of the mobile phases were tested and the desired resolution was achieved as hexane: ethyl acetate (9:1).

Calibration curve of standard was obtained by plotting peak areas versus concentration applied (Fig 1). It was found to be linear in the range of 1-5 µg/spot. Equation of the calibration curve was y = 3096x + 4265. The correlation coefficient was 0.976 and thus exhibited good linearity between concentration and area. Scrutiny of tested extracts explored that the acetone extract was more rich (151.0 µg/gm) in this bioactive compound whereas methanol extract contained least amount (29.34 µg/gm) of it. HPTLC quantification details are indicated (Fig 2, 3, 4). The results obtained by HPTLC were in accordance with the amount collected by column chromatography. This is an accurate, simple, reproducible and reliable method for quantitative estimation of components from plant extracts.

CONCLUSION

Juglone (5- Hydroxy 1, 4- Naphthaquinone) had been isolated from the acetone extract of *J. regia* stem bark. This biologically active compound was estimated quantitatively from various bark extracts by HPTLC method. The proposed method was found to be simple, rapid, accurate, precise, economic, reliable and reproducible.

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