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Estimation of Quercetin, an Anxiolytic Constituent, in *Elaeocarpus ganitrus*

Balbir Singh^{*1}, Mohan Pal S. Ishar¹ and Anupam Sharma²

1. Pharmacognosy and Phytochemistry Laboratory, Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar-143 005, India [E-mail: balbir_gndu@yahoo.com]
2. University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh-160 014, India

Having established quercetin as the anxiolytic constituent of *Elaeocarpus ganitrus*, it was decided to use it as marker to standardize the plant material. Quercetin was used as an external standard for determining its content in *E. ganitrus* beads by TLC densitometry. An HPTLC densitometric method has been developed to estimate quercetin in *E. ganitrus* beads so that plant can be standardized on the basis of its bioactive marker. Two methods were followed for preparing the test samples for determining the quercetin content. Initially, quercetin was determined in the ethanol extract of the plant material. It was also determined in the acid hydrolyzed ethanol extract, in order to free quercetin from its O-glycoside. Quercetin content in the hydrolyzed ethanol extract (0.11% w/w) of *E. ganitrus* beads was found to be about 4 times more than in the ethanol extract prepared by direct method (0.03% w/w). Results showed that quercetin occurs in *E. ganitrus* beads in the form of glycoside

Keyword: Anxiolytic, Quercetin, HPTLC Densitometry, *Elaeocarpus ganitrus*

1. Introduction

Genus *Elaeocarpus*, which has about 360 species, occurs throughout Australia, East Asia, Malaysia and the Pacific Islands. About 120 species belonging to this genus have been reported from different parts of Asia and out of this, 25 species occur in India alone^[1]. *Elaeocarpus ganitrus* Roxb. (Syn. *E. sphaericus* Gaertn., *E. angustifolius* Blume) is an evergreen tree, ripe fruits of which contain a hard and highly ornamental stony endocarp known as bead or nut, and is commonly termed as Rudrakasha in India. It holds popular belief reinforced by experiments that it has confirmed medicinal uses apart from its attractive stones^[2].

Ethnic people use fruit of *E. ganitrus* to treat various ailments. The flesh or pulp of drupe in green and fresh state is sour in taste, stimulates

appetite^[3] and is given in epilepsy, diseases of the head and mental illness. The fruit stone (seed kernel) is sweet, cooling and emollient. Externally the stone (fruit or drupe) is rubbed with water (like sandalwood) and then it is applied to small-pox eruptions. Similarly, it is applied on organs having burning sensation and in other conditions i.e. eruptions, measles, fevers, etc^[3].

E. ganitrus fruits contain glycosides, steroids, alkaloids and flavonoids. Apart from this, it has been found that the exocarp of the fruit supplies a nutritious reward to consumers, particularly rich in carbo-hydrates (21.0% dry mass, or 0.58 g per fruit) and proteins (4.3% dry mass, or 0.12g per fruit), but lacking in lipids^[4].

The leaves of *E. ganitrus* contain quercetin, gallic

and ellagic acids^[5]. Constituents such as (+)-elaecarpiline, isoelaecarpiline, (-)-epi-elaecarpiline, (+)-epi-isoelaecarpiline, (+)-epialloelaecarpiline, (-)-alloelaecarpiline, elaecarpidine, and pseudoepi-isoelaecarpiline having a dihydro- γ -pyrone chromophore in their molecules have also been reported. A minor non-aromatic indolizidine alkaloid, rudrakine (C₁₆H₂₃NO₃, mp 159-160°C), was also isolated from the leaves of *E. ganitrus* Roxb.^[6].

In most biological studies undertaken, extracts of *E. ganitrus* exhibited wide range of pharmacological activities and are identified as active against specific biological targets during large scale screening of multiple plant extracts. Literature reports are available on various pharmacological activities which include analgesic and anti-inflammatory^[7], CNS activities, typical behavioral actions^[8], sedative, tranquillizing^[8], hypnosis potentiation, antidepressant, antiasthmatic, hydrocholeretic^[8], antidiabetic^[9], cardiostimulation, antihypertensive^[10], anticonvulsant^[11], etc.

Despite a long history of use of *E. ganitrus* as a traditional medicine for the treatment of various ailments, especially CNS disorders, the plant has never been subjected to detailed antianxiety activity studies. Recently, authors have reported that among various extracts, viz., petroleum ether, chloroform, ethanol, and water, of *E. ganitrus* fruits, only the ethanol extract (200 mg/kg, *p.o.*) exhibited significant antianxiety activity on elevated plus maze model^[12]. An anxiolytic constituent quercetin was isolated from ethanol extract of *E. ganitrus* beads using antianxiety activity guided fractionation.

Present investigation was undertaken with an objective to develop an HPTLC densitometric method to estimate quercetin in *E. ganitrus* beads so that plant can be standardized on the basis of its bioactive marker.

2. Materials and Methods

2.1 Plant Material

Plant material was procured from Rati Ram Nursery, village Khurrampur, district Saharanpur, U.P, India, in the month of September-October 2007. The taxonomic identity of the plant was confirmed by Mr. Ram Prasad, Department of Botanical and Environmental sciences, Guru Nanak Dev University, Amritsar. A voucher specimen *Elaeocarpus ganitrus* (S.R. Bot Sci/0348) has been deposited in the same department's herbarium.

2.2 TLC densitometry

TLC densitometer system comprised of LINOMAT – 1V applicator and CAMAG TLC SCANNER – III with CATS 4 software. Chromatographic analyses of the standard and extracts were performed on pre-coated silica gel aluminum-based plates (20×20 cm, 0.2 mm, E Merck). Aliquots of standard and extracts were applied on TLC plates using 1.0 or 2.0 μ l CAMAG capillaries. TLC plates were developed using toluene:ethyl acetate:formic acid (10:3:1) as the mobile phase. The development distance was around 8 cm. The plates were dried and scanned at 372 nm.

2.3 Preparation of standard solution

Stock solution of quercetin (8 mg/10 ml) was prepared in ethanol. From this stock solution, five working standard solutions (6, 4, 3, 2 or 1 mg/10 ml) were obtained by appropriate dilution with ethanol.

2.4 Preparation of test samples

2.4.1 Direct method

Dried powdered beads of *E. ganitrus* (2 g), packed in a filter paper sachet, were defatted by refluxing in 250 ml round bottom flask on boiling water bath with 3×50 ml quantity of petroleum ether (1h each). The marc obtained was air dried and refluxed under similar conditions with 3×50 ml quantity of ethanol. The ethanol extracts were pooled, filtered and concentrated under reduced pressure. Dried ethanol extract was reconstituted in ethanol, in a volumetric flask, and its volume was made upto 10 ml.

Table 1: Quercetin content in *E. ganitrus* beads as determined by TLC densitometry.

Method	BS ₁ content (% w/w) (Mean ⁿ ± S.D.)
Direct extraction with ethanol	0.03 ± 0.001
After acid hydrolysis of ethanol extract	0.11 ± 0.001

n = 3

Table 2: Intra-day and Inter-day precision of Quercetin.

Marker	Concentration (ng/spot)	Intra-day precision (% RSD)	Inter-day precision (% RSD)
Quercetin	200	0.48	0.41
	400	0.53	0.65
	800	0.52	0.56

Table 3: Recovery study of Quercetin.

Marker	Amount of marker present (µg)	Amount of marker added (µg)	Amount of marker found (µg) (Mean ± S.D.)	Recovery (%)	Average recovery (Mean ± S.D.)
Quercetin	400	200	591.2 ± 5.64	98.53	98.32 ± 0.1
	400	400	786.2 ± 6.22	98.28	
	400	500	883.4 ± 7.84	98.16	

2.4.2 Acid Hydrolysis

Following the above procedure (Section 2.4.1), ethanol extract of *E. ganitrus* beads was prepared. Dried ethanol extract was heated in a 50 ml round bottom flask with 6% aqueous hydrochloric acid (25 ml) for 45 min on water bath. Aglycones, precipitated on cooling the solution, were removed by filtration, and dissolved in ethanol. The filtrate was fractionated with 3×20 ml quantity of diethyl ether; the fractions were combined, dried over anhydrous sodium sulphate, and the solvent was removed completely under reduced pressure. The diethyl ether extract, thus obtained, was pooled with ethanol solution of aglycones. Finally, volume was made up to 10 ml with ethanol in a volumetric flask.

2.5 Preparation of Standard Curve

Two µl each of the stock solution and the four working standard solutions of quercetin were applied, in triplicate, on 20 cm × 20 cm TLC plates. The plates were developed using toluene:ethyl acetate:formic acid (10:3:1) as mobile phase. The developed plates were scanned at 372 nm using

TLC densitometer. A standard graph was plotted against absorbance and quercetin amount (µg).

2.6 Estimation of Quercetin Content in Test Samples

Aliquots (2 µl) of test samples were applied, in triplicate, to the TLC plates. The plates were developed using toluene:ethyl acetate:formic acid (10:3:1) as mobile phase, and the chromatograms were scanned at 372 nm using TLC densitometer. The area under the curve of every sample was recorded, and quercetin content was determined from the regression equation of the standard graph.

2.7 Validation of TLC Assay

ICH guidelines were followed for the validation of the analytical method developed (CPMP/ICH/281/95).

2.7.1 Instrument Precision

Instrumental precision was checked by repeated scanning (n=7) of the same spot of quercetin (400 ng/spot), and was expressed as relative standard deviation.

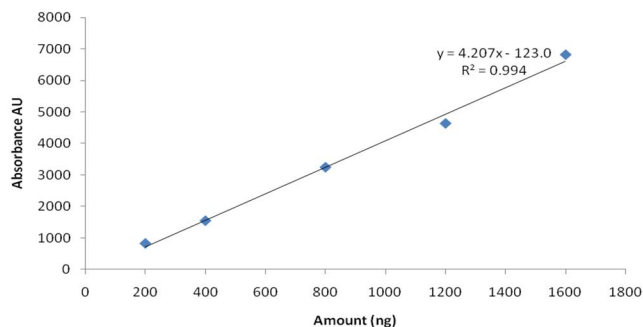


Figure 1: Standard curve of absorbance against amount of Quercetin.

2.7.2 Repeatability

The repeatability of the method was confirmed by analyzing 400 ng/spot of quercetin individually on a TLC plate (n=5) and expressed as %RSD.

2.7.3 Intra-Day and Inter-Day Variation

Variability of the method was studied by analyzing aliquots of standard solution containing 200, 300 or 400 ng/spot of quercetin on the same day (intra-day precision) and on different days (inter-Day precision).

2.7.4 Limit of detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined by applying different concentrations of the standard solutions of quercetin along with ethanol as blank and determined on the basis of signal-to-noise ratio

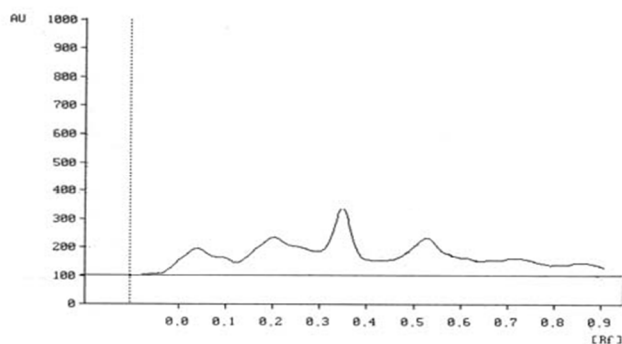


Figure 2: TL chromatogram of ethanol extract of *E. ganitrus* beads

(S/N). LOD was determined at S/N of 3:1 and LOQ at S/N of 10:1.

2.7.5 Recovery Studies

The accuracy of the method was assessed by performing recovery studies at three different levels (50, 100 and 125% addition of quercetin). The percent recovery and average percent recovery were calculated.

2.7.6 Specificity

This was ascertained by analyzing the standard compound and sample. The band for quercetin from sample solutions was confirmed by comparing the R_f and spectra of the bands to those of the standards. The peak purity of quercetin was analyzed by comparing the spectra at three different levels, i.e., start middle and end positions of the bands.

3. Results and Discussion

The developed plates were scanned at 372 nm using HPTLC densitometer. Figure 1 shows standard plot of quercetin. Figures 2 and 3 show TL chromatogram of ethanol extract of *E. ganitrus* and that of acid hydrolyzed ethanol extract of *E. ganitrus*. Table 1 shows quercetin content in *E. ganitrus* beads as determined by TLC densitometry. Results of intra-day and inter-day precision, and recovery studies of quercetin are shown in tables 2 and 3 respectively.

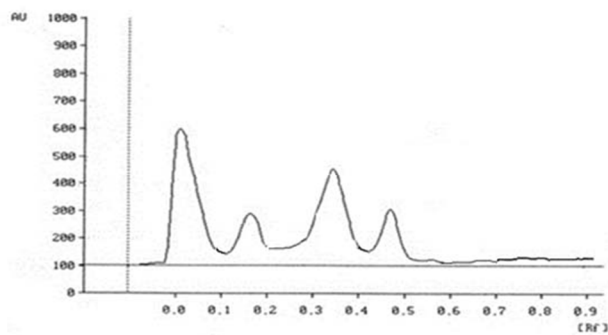


Figure 3: TL chromatogram of acid hydrolyzed ethanol extract of *E. ganitrus* beads.

Having established quercetin as the anxiolytic constituent of *E. ganitrus*, it was decided to use it

as marker to standardize the plant material. Quercetin was used as an external standard for

determining its content in *E. ganitrus* by TLC densitometry. TLC was used as its method of development is easy, cost effective, efficient and requires not much clean-up of the test samples.

TLC studies revealed that quercetin resolved well in test samples using toluene:ethyl acetate:formic acid (10:3:1) as the mobile phase. The chromatograms of quercetin and acid hydrolysed ethanol extract of *E. ganitrus* beads were scanned under UV at 372 nm (Figures 2 and 3).

It is a well-known fact that quercetin occurs in plants mainly as glycoside. Quercetin has been isolated, in the present investigation, as an aglycone. Keeping in view the possibility of its occurrence in the plant as glycoside, extraction procedure was modified for preparing the extract for determining quercetin content.

Standard procedures were adopted to obtain ethanol extract of *E. ganitrus* beads. Two methods were followed for preparing the test samples for determining the quercetin content. Initially, quercetin was determined in the ethanol extract of the plant material. It was also determined in the acid hydrolyzed ethanol extract, in order to free quercetin from its O-glycoside. Quercetin content in the hydrolyzed ethanol extract (0.11% w/w) of *E. ganitrus* beads was found to be about 4 times more than in the ethanol extract prepared by direct method (0.03% w/w) (Table 1). From this observation, it can be concluded that most of the quercetin is present in O-glycosidic form in *E. ganitrus* beads.

Further, the TLC assay was validated in terms of precision, repeatability and accuracy. The linearity of the calibration curve was achieved between 200-1600 ng for quercetin ($r^2 = 0.994$; Figure 1). The TLC assay was found to be precise with % RSD for intra-day in the range of 0.48-0.52 and inter-day in the range of 0.41-0.56 for different concentrations of quercetin (Table 2). This indicates that the proposed method is precise and reproducible. The LOD and LOQ values for quercetin were found to be 60 and 140 ng/spot respectively. The average recovery at three different levels of quercetin was observed to be very high (98.32%) (Table 3) indicating that the assay procedure developed, in the present investigation, is quite reliable.

4. Acknowledgements

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