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RESEARCH ARTICLE

Validated method for simultaneous quantification of gallic acid and quercetin in *Acacia catechu* (L.f.) Willd

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Abstract: The present study was undertaken with the aim of establishing a validated method for the estimation of Quercetin and Gallic acid in *Acacia catechu*, a traditionally used antidiabetic species. HPTLC instrument consisted of a Linomat-5 applicator (CAMAG, Muttenz, Switzerland) fitted with a Linomat-5 automatic sample applicator and CAMAG TLC scanner-3 ("Scanner_180710" S/N 180710 (2.01.02)) run by WinCa ATS software (version: 1.4.6.2002). The linearity range was 0.5-2.0ug for both Quercetin and Gallic acid. The correlation coefficient was 99.4% and 99.0% for Quercetin and Gallic acid, respectively, and the mean recovery was 100.3 and 99.92 for Quercetin and Gallic acid. This simple, precise, and accurate method provided good resolution of other constituents present in the extract. This validated method can be successfully applied for evaluation of various formulations containing *Acacia catechu,* besides lending support to its traditional use.

Keywords: *Acacia catechu*, HPTLC, Quercetin and Gallic acid.

Introduction:

In virtually all cultures, medicinal plants have been used for curative and healing purposes. Nearly 80-85% of the population in developing nations resort to traditional /plant-based medicines for primary health concerns (Hao and Xiao, 2020). 'WHO Traditional Medicine Strategy 2014–2023' exhorts upon an amalgamation of traditional medicine with conventional medicine to achieve health for all (Sen et al., 2017). Plants serve as nature's pharmacy as they contain many secondary metabolites like phenolics, tannins, flavonoids, and alkaloids, which are bioactive and exert synergistic effects on human health (Chinsembu, 2019). Plant-based natural products are the main targets for the identification of lead molecules that may have promising effects in the programs for the development of drugs (Salehi et al., 2019). Despite the availability of myriad oral anti-diabetic drugs in the market, the management of diabetes without complications still eludes. Conventional medicine does help in maintaining glucose levels, but underlying metabolic and glucoregulatory disturbance is not fixed. The continued use and popularity of plant-based traditional medicine require scientific validation of therapeutic properties to contribute more novel molecules for the management of Type 2 diabetes. In recent times, HPTLC fingerprinting has emerged as a vital and efficient tool for the identification of the bioactive principles of medicinal plants.

Acacia catechu is a deciduous thorny tree up to 15–17 m tall. The species is native to central and east Africa, South Asia, Bhutan, China, India, northern and north-western Pakistan, Myanmar, and Nepal (Patel *et al.*, 2009). It is found extensively at an elevation of 1200 meters throughout India in the lower Shiwaliks of Jammu and Kashmir, Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, West Bengal, and Sikkim.

Acacia catechu finds traditional usage in many cultures in Asia. Bark decoction is used against cough, cold, and diarrhea (Singh and Lal, 2006). Khoyer, a concentrated extract made by boiling the dark heartwood is used for pain relief and blood glucose control (Rahmatullah *et al.*, 2013). Ayurvedic skin tonic Khadira Rishta is prepared from heartwood. Chinese use heartwood extracts called 'Ercha' for cough, diarrhea, skin ulceration, and lesions (Shen *et al.*, 2006). Soft branches used as chew sticks globally owing to antibacterial properties (Shen, 2006). Used in asthma, mouth sores, chest pain, bronchitis, cancer, diarrhea, sore throat, ulceration, healing of wounds, and vitiligo. Known to exert antifungal, spasmolytic, and antiviral (Alamabayan et *al.*, 2015).

2. Material and Methods

2.1 Sample Collection

Heartwood of *Acacia catechu* was collected from Kathua (32.3863ºN, 75.5173ºE) and was authenticated by Dr Nitin, Curator, Department of Botany, University of Jammu, vide voucher number HBJU-16704.

2.2 Sample Preparation

Chips of *Acacia catechu* heartwood were thoroughly washed with water to remove adhering soil and other surface contaminants. The materials were subsequently shade-dried for several days under well-ventilated conditions. The dried plant materials were finely powdered and passed through a 120-mesh sieve to obtain a uniform particle size. The powdered plant material was subjected to maceration in ethanol for 48 hrs. The maceration process was repeated for thrice. The resulting extracts were filtered, and the combined filtrates were concentrated under reduced pressure using a rotary evaporator at a temperature below 45 °C to obtain the crude ethanolic extracts (Bhatia et al., 2019).

2.1 High-Performance Thin Layer Chromatography (*HPTLC)*

2.1.1 Chemicals and reference compounds

All reference compounds viz. gallic acid and quercetin (97% pure) were procured from Sigma-Aldrich (USA). All reference compounds were stored at -20 °C. All solvents used in our experimentation were of HPTLC grade that was purchased from S.D. Fine Chemicals, Mumbai, India.

2.1.2 High-Performance Thin Layer Chromatography (HPTLC) Instrumentation

HPTLC instrument consisted of Linomat-5 applicator CAMAG (Muttenz, Switzerland) fitted with Linomat-5 automatic sample applicator and CAMAG TLC scanner-3 ("Scanner_180710" S/N 180710 (2.01.02)) run by WinCATS software (version: 1.4.6.2002). The stationary phase consisted of pre-coated silica gel 60 F254 TLC plates (20 × 10 cm, E. Merck, Darmstadt, Germany). Each sample was placed at the plates as bands 6 mm wide, with 13 mm distance between tracks, via a Linomat-5 automatic sample applicator fitted with a 100µl Hamilton syringe. The dosage speed from the Hamilton syringe was 150 nL/s. Conditions desired for densitometric scanning were fixed at 4.00 x 0.30 mm slit dimension with a scanning speed of 20 mm/s and data resolution of 100 µm/step. The HPTLC was performed at 24 ±2 °C temperature with 45% relative humidity in CAMAG twin trough glass chamber (20 cm x 10cm) saturated before for 20 minutes with mobile phase vapor.

2.1.3 Simultaneous quantification of gallic acid and quercetin

5 µL of plant sample and reference compounds were applied on an HPTLC plate and were developed in mobile phase-toluene: ethyl acetate: formic acid (13.5:9:0.6 v/v/v). After development up to 75 mm, plates were dried with a hot air dryer, and clear bands were visualized under UV (UV cabinet with dual-wavelength UV lamp. Immediately, plates were scanned at 254 nm and 366 nm reflectance wavelengths.

3. Results & Discussion

3.1 HPTLC method development for simultaneous quantification of polyphenolic compounds (Gallic acid and Quercetin)

3.1.1 Validation of HPTLC Methods

HPTLC method validation was performed on parameters such as linearity, limit of sensitivity, specificity, precision, accuracy, recovery, and robustness according to the ICH guidelines (International Council for Harmonisation, 2005).

3.1.2 Precision

The same spot of gallic acid (500 ng/spot), quercetin (500 ng/spot), with n=7, was used to check the accuracy of the instrument. Repeatability (intraday) and reproducibility (inter-day) of the method were implemented with three different concentration levels of reference compounds on the same day and also analyzed after 3 days, respectively. These analyses were attempted seven times, and all the obtained results are represented as mean ± % RSD.

3.1.3 LOD and LOQ

Signal-to-noise (S/N) ratios with different concentrations of reference compounds were applied along with methanol as a blank and evaluated LOD and LOQ values. LOD was measured as 3,1 (SD/S), whereas limits of quantification (LOQ) were 10.1 (SD/S), where S represents the slope, and SD means the standard deviation of the Y-intercept from the regression line.

3.1.4 Specificity

Peak purity was assessed by comparing standard reference compounds with those of plant sample at the beginning, peak maxima, and end of the peak. Additionally, Overlaying spectra of the isolated bands from the plant samples were compared with the marker compounds. The segregated bands of each reference standard compound were compared with plant samples and were checked for corresponding Rf values in their scanned densitometric chromatograms.

3.1.5 Method Development

HPTLC technique was used for the quantitative assessment of polyphenols, i.e., gallic acid and quercetin in heartwood of *Acacia catechu.* HPTLC studies showed that the solvent system for simultaneous quantification of gallic acid and quercetin was achieved with tolune: ethyl acetate: formic acid (13.5:9:0.6 v/v/v/v) mobile phase that gave well-resolved spots at Rf 0.24 and 0.52 for gallic acid and quercetin, respectively (Table 1). Well-resolved spots for gallic acid and quercetin reference compounds were visualized at 254 nm and 366 nm without any post-derivatization treatment.

Table 1: Method validation for Gallic acid and Quercetin quantification in *Acacia catechu* heartwood

Parameters	Gallic acid	Quercetin
Linearity range	0.5-2.0 µg	0.5-2.5 µg
Correlation coefficient	99.4%	99.0%
Regression equation	area=16032+10655 gallic acid (ug/spot)	area=40741+4002quereci tin conc. (ug/spot)
Calculated SD Value		
a. Limit of detection (LOD) (ng) [3×SD/S]	30	40
b. Limit of quantification (LOQ) (ng) 6[10×SD/S]	90	120
Rf	0.24	0.52
Precision	0.4	0.52
Intraday RSD (%), n=7	0.45	0.53
Interday RSD (%), n=7	0.47	0.55
Recovery (%) Mean Recovery (%)	100.3	99.92

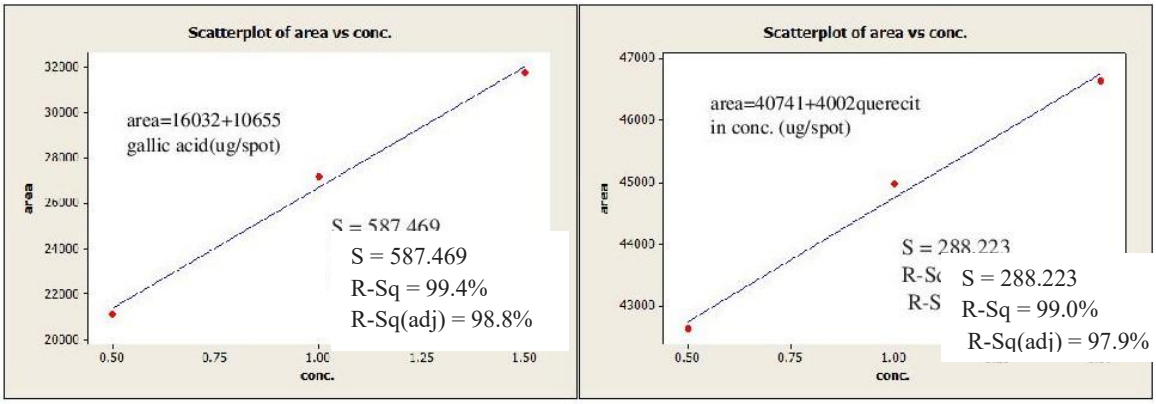

Figure 1: The linearity graph of the standard Gallic acid and Quercetin

HPTLC-Densitogram patterns obtained for the *Acacia catechu* heartwood sample and standard compounds depicted that the peaks corresponding to Rf 0.24 and Rf 0.52 were superimposable in all the test samples (Fig. 4.21(a-c)). The spectrum characteristics corresponding to this peak were also found to match exactly, indicating the compound corresponding to Rf of the standard and the test samples to be identical. Linearity of the calibration curve was achieved between 0.5-2.0 µg for Gallic acid, whereas 0.5-2.5 µg range showed good linearity for quercetin (Figure 1(a-b). The phytochemical screening of *Acacia catechu* was based on Gallic acid and Quercetin. HPTLC was used for the quantitative estimation of Gallic acid and Quercetin in the *Acacia catechu* heartwood sample. Gallic acid and quercetin from the extracts of *Acacia catechu* were confirmed by matching their single spot at Rf = 0.24 and 0.52 values with the peaks of standards (Fig. 2(a-c)).

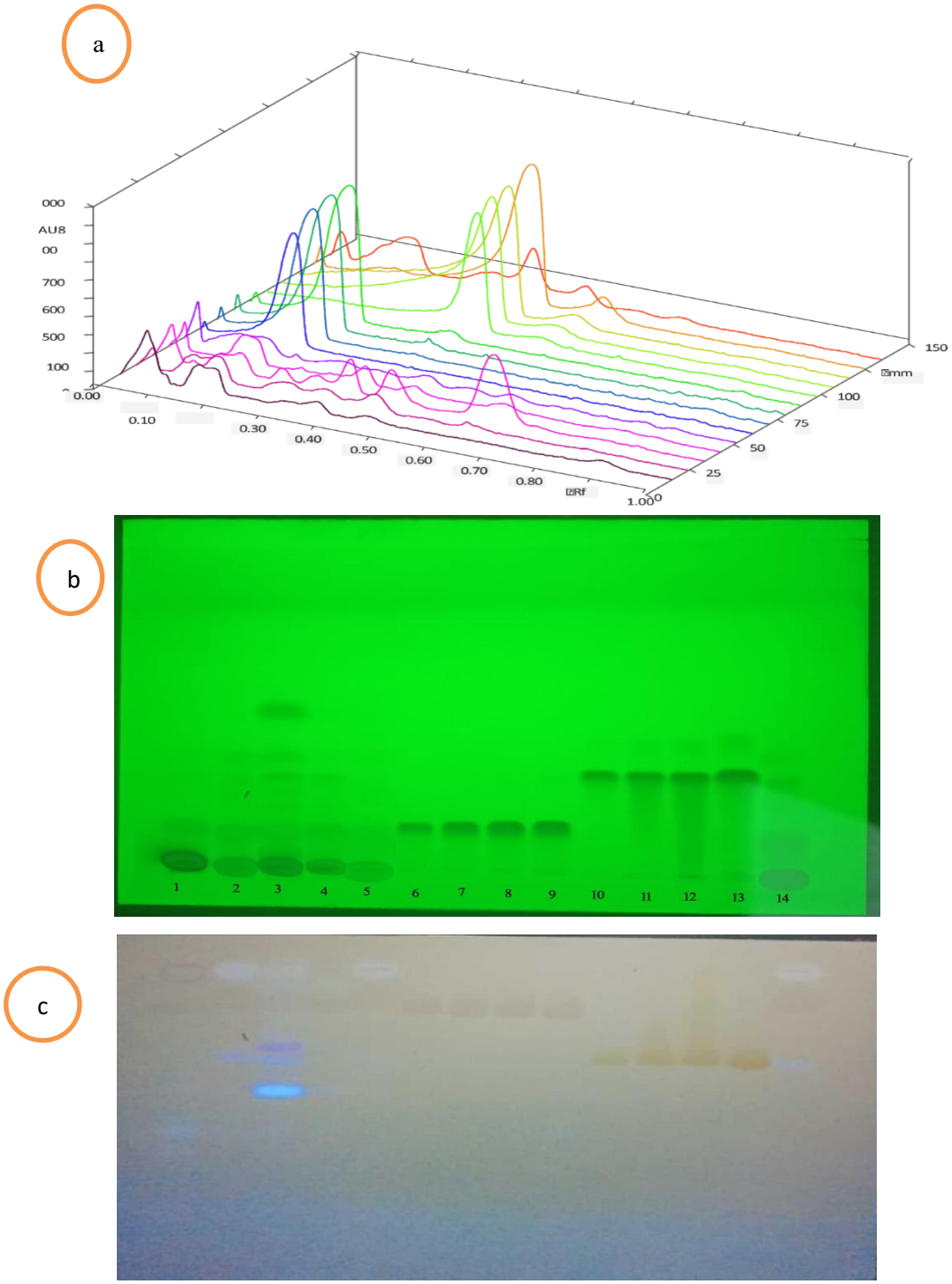

Figure 2(a-c): 3-D Chromatogram (a) HPTLC Fingerprinting of simultaneous quantification of gallic acid and quercetin standards along with *Acacia catechu* heartwood.

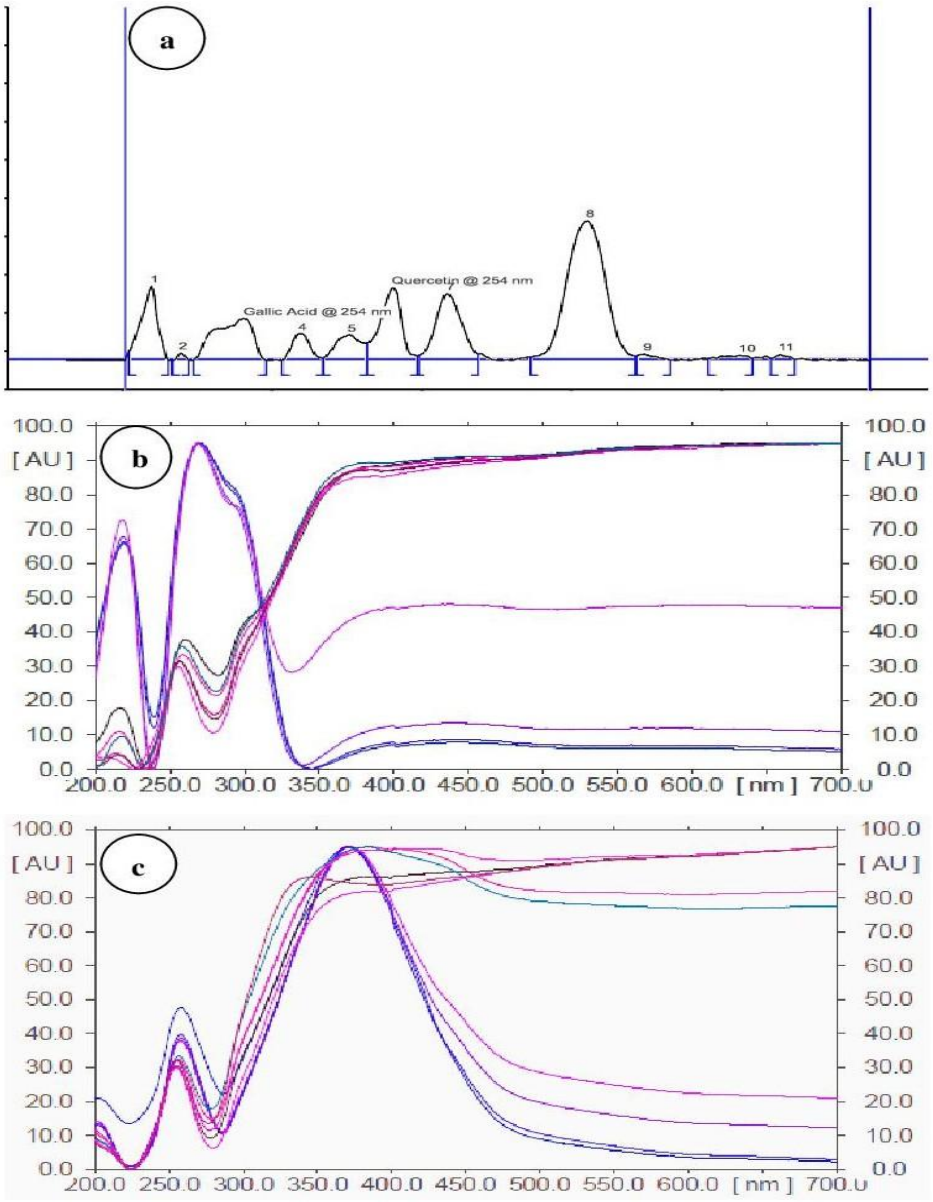

Figure 3(a) Densitogram of Quercetin and Gallic Acid with Acacia catechu sample (b--c) Overlay spectra of Gallic acid and Quercetin with *Acacia catechu* heartwood sample at 254 and 366 nm.

The quantity of Gallic acid and Quercetin in *Acacia catechu* heartwood was evaluated by applying the linear regression equation, and the content was displayed in Table 2.

Table 2: Quantification of Gallic acid and Quercetin in *Acacia. catechu* heartwood

Plant	Part used	%age conc. of Gallic acid	%age conc. of Quercitin
Acacia catechu	Heartwood	0.018	0.00463

The HPTLC method developed and validated for the simultaneous estimation of Gallic acid and Quercetin happens to be simple, sensitive yet precise, and can be suitably used for the quantification of Polyphenolics in plant extracts. Results obtained in our study imply that the choice of plants by folklore for diabetes does have a relevant scientific basis. Polyphenolics have been evidenced in several studies to ameliorate diabetic complications besides improving beta cell function and insulin resistance. Quercetin is reported to promote beta cell proliferation and insulin secretion, regulate AMPK which in turn enhances GLUT-4 expression and translocation, and regulate inflammatory pathways nuclear factor kappa B (NF-κB) and various interleukins (Iskender *et al.,* 2017). Gallic acid also upregulates PPARγ and AMPK expression thereby improving glucose metabolism (Variya *et al.,* 2020) and depresses ER stress and apoptosis of beta cells; decline in interleukin levels and NFKβ thus suppressing inflammation, a major contributor to insulin resistance (Obafemi *et al.,* 2021). Quantification of these polyphenolics thus yields a basis for the traditional usage of them for controlling blood glucose levels.

Declarations

Competing interests: The author has no competing interests to disclose.

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