Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 566-571
Received: 10-05-2020
Accepted: 12-06-2020

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Investigations into the phytochemical profile in the seeds of *Elaeocarpus variabilis* fruits: A potential untapped source endemic to Western Ghats

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DOI: https://doi.org/10.22271/phyto.2020.v9.i4h.11762

Abstract

Elaeocarpus variabilis, a highly endemic plant to the Nilgiris belonging to the family of *Rudraksha* owns a whole lot of phytochemical properties such as antioxidant, anti-inflammatory, antimicrobial and anticancer activity. Despite its enormous properties, it is one of the unexplored herb available locally. The seeds showed such immense properties and the identification of compounds responsible for these properties is a crucial part. Hence, the qualitative identification was carried out using Thin Layer Chromatography (TLC) and normalization of the appropriate solvent system for quantification by Preparative Thin Layer Chromatography and composition of the compounds present were investigated using High performance thin layer chromatography (HPTLC). The results from HPTLC showed the presence of quercetin like compounds and further, structural elucidation of the active principle can be proceeded with Liquid Chromatography-Mass spectrometry (LCMS) and to determine the composition of the phytochemicals present.

Keywords: Active principle, *Elaeocarpus variabilis,* chromatography, quercetin

Introduction

Southern India serves as a home for many diverse species of plants and animals. Western Ghats is a hotspot serving as a habitat for many endangered species of flora and fauna which have strong medicinal and therapeutic properties. Creating awareness on these fruits and their pharmaceutical values can create a great impact on the medicinal era providing more scope on their value added products [1]. Wild fruits are available in plenty in their natural habitats and still their germplasm needs to be conserved and one such species is *Elaeocarpus variabilis. Elaeocarpus* is a genus which belongs to the family, *Elaeocarpaceae* (commonly termed as *Rudraksha* in India) with evergreen broad-leaved trees and shrubs widely distributed in warm tropical regions across the globe. *Elaeocarpus variabilis* is a 40-meter-tall evergreen tree with ripening seeded fruits which contain a hard and stony endocarp known as bead or nut [2, 3].

Most recent studies indicate the presence of chemical constituents such as alkaloids, flavonoids, glycosides, fatty acids, tannins, triterpenes, steroids, saponins, ellagic acid derivatives and cytotoxic compounds in *Elaeocarpu* species with traits to cure different ailments like headache, fever, chicken pox, mental disorders, burn or pox marks and also to wound healing [4, 7]. The therapeutic activity of *Elaeocarpus* such as anti-asthmatic, anxiolytic, anti-depressant, anti- diabetic and various antioxidant, antimicrobial, anti-mutagenic, anti-inflammatory, anti-depressant, anti-carcinogenic, anti-allergic were also studied [8, 13]. *In vivo* and *invitro* studies showed the best antioxidant activities on cold extracted sample with maximum presence of phenols and flavonoids to identify the best extraction system, by comparing with, decoction, maceration, percolation, squeezing and soxhlet extractions [14, 18].

The isolation, purification and identification of the active principle ingredients from the plant extract have always been a tedious and challenging task [19]. Among which, thin layer chromatography was identified to be a rapid method used for the qualitative identification of the bioactive composition. Retention factor of the clear spot is calculated to compare and standardize the presence of phenols, flavonoids and amino acids in the plant samples. Majorly silica gel, and other materials like cellulose are also used as stationary phase to achieve the maximum separation of bands, thereby indicating the separation of the various compounds using the identified solvent system [20]. These TLC and HPTLC silica coated plates were viewed under absorption– reflection detection mode at different wavelengths say, 366nm and 425nm [21, 23]. Hence, HPTLC proves to be time efficient method to identify multiple target compounds such as flavonoids, polyphenols using a common standard and it also proves to be

a cheaper method to face the analytical problems in the plant based studies [24, 25]. The antioxidant constituents of plant extracts were effectively isolated using advanced chromatographic techniques and studied using Liquid Chromatography coupled with Mass spectrometry for their structural elucidation and quantification [26, 27].

The aim of the project is to identify and isolate the active principle from the seeds of *Elaeocarpus variabilis* responsible for the various activities such as antioxidant, anti-inflammatory, anti-microbial, anti-tuberculosis properties and to perform Thin Layer Chromatography (TLC) and Preparative Thin Layer Chromatography (PTLC) followed by standardization and identification of the active compound by High Performance Thin Layer Chromatography (HPTLC) and finally Liquid Chromatography coupled with Mass Spectrometry (LCMS) for the determination of active ingredient composition.

Materials and Methods

Plant Sample Collection

Elaeocarpus variabilis plants with the fruits were collected from in and around Nilgiris district along the Western Ghats and the whole plant was authenticated by Scientist In-Charge, Botanical Survey of India, Tamil Nadu Agricultural University (TNAU), Coimbatore. The voucher specimen of the plant sample is deposited in the herbarium of the department.

Preparation of Seed and Pulp extract

Cold Extraction

The seed sample of *Elaeocarpus variabilis* was dried under shade and ground to coarse powder. Cold extraction of the seed was performed by using the polar solvent ethanol [28]. 100ml of the solvent was used to extract with 10 gram of powdered sample each by keeping it in an orbital shaker for over 24hours [29]. This process is repeated for five batches and the extractwas collected and stored in brown bottles. The extracts obtained were concentrated under natural evaporation under shade, which was then stored for further experiments. Yield % can be calculated based on the following formula,

$$\text{Yield \%} = \frac{\text{Dry weight of the extract}}{\text{Dry weight of the plant sample}} * 100$$

Thin Layer Chromatography

Properly washed and clean micro slides (75mm x 25mm) were wiped with 70% ethyl Alcohol using non-absorbent cotton and kept for drying in the hot air oven. Silica gel suspension was prepared with distilled water in a glass reagent bottle and the slurry was poured uniformly on the Micro slide and ensured that it has a uniform thickness. The plates were then dried and stabilized at 70 °C for 1 hour in the hot air oven. The TLC chamber was filled with solvent system and sealed tightly to prevent the evaporation of solvent. The sample was spotted using the capillary tubes at the bottom of the plate, roughly 1cm from the bottom of the slide. The TLC plate is immersed into the TLC chamber containing the solvent mixture (Figure 1). Once the spots are developed, the Solvent Front is noted and the plated are dried. The sample spots can be observed under a Visible, UV (365 nm), Short UV (254 nm) by placing in the UV Cabinet. Revealing of compounds can be done using the iodine chamber by adding a few grams of dry silica gel powder to cover the bottom along with 5-10 iodine crystals. Further, visualization using Iodine Chamber to detect the spots if they are not visible under UV light are performed. The distance travelled by the solvent, solvent front is noted and the distances of spots seen at different points are also to be measured, to calculate the Retention Factor (R_f).

$$\text{Retention Factor} = \frac{\text{Distance moved by the compound above origin (cm)}}{\text{Solvent front (cm)}}$$

Fig 1: Pictorial representation of Real-time TLC developing chamber

Preparative Thin Layer Chromatography

In preparative TLC, the identified compound which is separated as thin, long streaks can be recovered by scraping the sorbent layer at the particular region of interest from the plate and eluting the separated material from the sorbent using a strong solvent. The scaled up form of TLC includes a square glass plate (20cm x 20cm). As done there in TLC, the silica gel is prepared and the samples are spot on the plate in shape of small bands. Now, the PTLC plate in a bigger solvent TLC chamber containing the solvent mixture. Once the spots are developed, the Solvent Front is noted and the plated are dried. The sample spots can be observed in UV Cabinet under Visible, UV (365nm), short UV (254nm) wavelengths and destruction visualization using Iodine Chamber were performed (Figure 2). The Retention Factor (R_f) is calculated. The identified spot is then scraped out from the scribed area and the collected powder is stored. Further, the residual adsorbent material was scraped out and the purified in a Bucher funnel tightly packed with glass wool and stored for futuristic studies.

Fig 2: Iodine Chamber used for the detection in Preparative Thin Layer Chromatography

High Performance Thin Layer Chromatography

HPTLC is an updated automated version of thin-layer chromatography (TLC) with high resolution to quantify and estimate the compounds of interest. HPTLC was performed on CAMAG Automatic TLC Sampler 4 (ATS 4) by dissolving the sample in 20ml of solvent. The dissolved sample was coated on the HPTLC plates Silica gel 60 F 254 (20cm x10 cm) and these plates were saturated and placed in the twin trough chamber. The developing system is the solvents standardized using Thin Layer Chromatography. The parameters on the device were checked for band length – 8mm, number of tracks – 15, first application position X – 20mm and application position Y – 8mm. Derivatization by automatic spraying is done by heating at 100°C for 5 minutes and 3.5ml of a solution of 1% NP reagent in Methanol/Ethyl acetate followed by 5% macrogol 400 in Methanol/Di-chloromethane is sprayed over it. The positive control used here is Quercetin. After 30 minutes, the plate is captured under UV at 366 nm.

Results and Discussion

The sample was collected and the pretreatment methods such as cleaning, drying and grinding were carried out successfully, which is the main precursor for the extraction process. The whole plants were authenticated by Scientist In-Charge, Botanical Survey of India, Tamil Nadu Agricultural University (TNAU), Coimbatore. The voucher specimen of the plant sample of *Elaeocarpus variabilis* was stored in the herbarium of the department (No.: BSI/SRC/5/23/2020/Tech/557)

Cold Percolation

By comparing the yield from hot and cold extracts of the seed, it was inferred that the heat based extraction could show deterioration of certain compounds like cholesterol, saponins, volatile oils, proteins and amino acids, alkaloids, flavonoids, cellulose, leuco-anthocyanidines, terpenoids present in *Elaeocarpus variabilis*. Hence, cold percolation method was preferred over, hot extraction using Soxhlet apparatus. The yield percentage was calculated to be 9.4% and 2.4% for methanol and acetone extracts respectively [30].

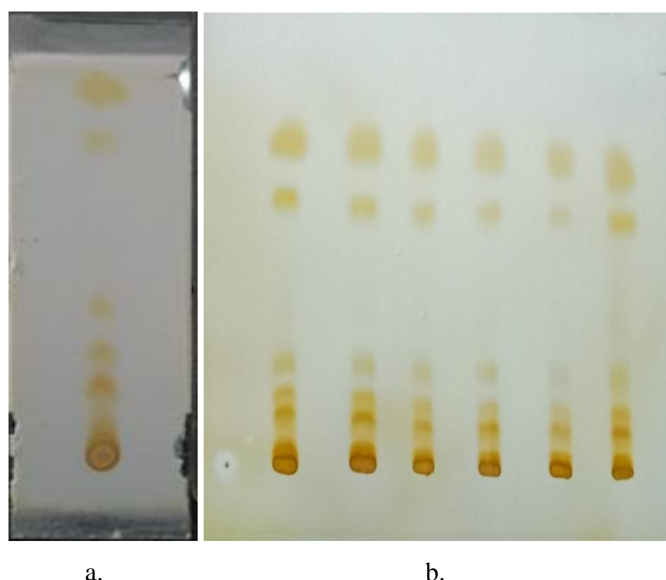
Thin Layer Chromatography and Preparative TLC

Thin layer Chromatography (TLC) is a primary technique used to qualitatively estimate the presence of the bioactive ingredients responsible for the various phytochemical properties expressed using Retention factor (R_f) to calculate the separation of the required compound. From Figure 3, an effective separation of 6 different compounds can be observed in the best solvent system, Hexane/ Ethyl Acetate/ Chloroform/ Toluene in the proportion of 81.52/4.07/9.97/5.43 which showed better resolution when compared to other systems. Hence, the separation of 6 bands out of which, 2 highly low polar compounds with the R_f of 0.81 ± 0.01 and 0.95 ± 0.01 were found closer to the solvent front and a highly polar compound, whose R_f was, 0.07 ± 0.01 and three main mid polar compounds 0.18 ± 0.01, 0.25 ± 0.01 and 0.38 ± 0.01 were observed. Based on the retention data obtained from the one-dimensional Thin Layer Chromatography by R_f and considering several factors like the resolution, number of compounds separated, and solvent system, the scaled up on a larger plate called Preparative Thin Layer Chromatography (PTLC) was carried out with the standardized solvent system, Hexane/ Ethyl Acetate/ Chloroform/ Toluene (81.52/4.07/9.97/5.43,v/v/v/v) after confirmed in Thin Layer Chromatography.

The Retention factor (R_f) was calculated in comparison with the Quercetin standard by concentrating on the polyphenols and sterols which contribute to the major share in the seeds of *Elaeocarpus*. The separation of the compounds and their corresponding Retention factor (R_f) were in accordance with the Retention Factor (R_f) obtained using Thin Layer Chromatography (Bhole *et al.,* 2015) [31].

Table 1: Thin Layer Chromatography results in terms of Retention factor with the number of compounds separated per plate with their appropriate solvent system

Mobile Phase with Solvent system (in percentage)	Number of compounds separated	Retention Factor (R_f value)
Hexane/ Ethyl Acetate/ Chloroform/ Toluene (81.52/4.07/9.97/5.43, v/v/v/v)	6	0.04 ± 0.02
		0.08 ± 0.02
		0.12 ± 0.02
		0.19 ± 0.02
		0.47 ± 0.02
		0.84 ± 0.02



a. b.

Fig 3a): Separation of the compounds using TLC in the trial batches, (b) Separationof the compounds on PTLC plate after standardization using TLC and PTLC

High Performance Thin Layer Chromatography

HPTLC is advantageous over other chromatographic techniques due to its ability to analyze its compound composition simultaneously using a very little mobile phase at a greater extent. HPTLC fingerprinting is used for the confirmation of the presence of the phytoconstituents in the sample. The declaration of the presence of the essential phytochemical can be made by height of the peak in the densitogram made in comparison with the standard. The area under peak and the R_f values also add to the confirmation of the results. Based on the values from Table 4, it can be concluded that quercetin like compounds are largely present in the extract confirmed by the peak height of sample compared to that of quercetin. The maximum area covered was found to be 5401.7, were the peak obtained is structurally identical with the maximum at 380nm [31]. Derivatization of the plates was done using 1% ethanolic aluminium chloride and visualization was done at two wavelengths, 254nm and 366nm before and after derivatization process. The regression

lines of the standard graph were plotted to identify the concentration of quercetin like compounds in the seed extract (Figure 4). In sample I, 19.34µg and in Sample II, around 22.50µg of the compound were quantified in 1mg of the sample kept under analysis [32]. The visualization of the Sample II (Toluene/ Ethyl Acetate/ Formic Acid - 54.35/43.48/2.17, v/v/v) plate before and after derivatization showed the presence of phenols, by emitting fluorescence at 366nm as shown in Figure 5 and 6.

Table 2: Peak table – showing the data obtained from the densitogram on the R_f values, Height and Area of the seeds of *Elaeocarpus variabilis* against the standard

S. No	Sample	Assigned Standard	R_f values	Height	Area
1	Sample I	Quercetin	0.08	156.5	4567.3
2	Sample II		0.58	294.8	5401.7

*Sample I – developing solvent is Hexane/ Ethyl Acetate/ Chloroform/ Toluene and Sample II – developing solvent is Toluene/ Ethyl Acetate/ Formic Acid

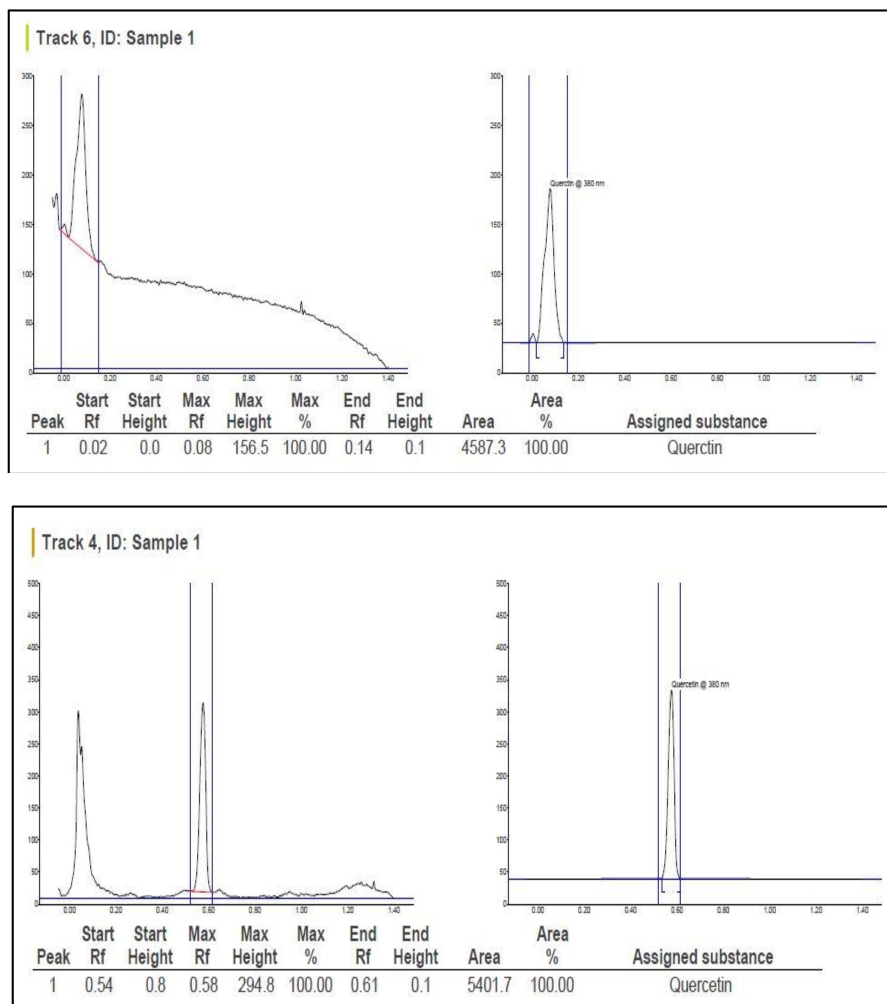


Fig 4: Densitogram of the Active principle in the Cold methanolic extract of the seeds of *Elaeocarpus variabilis* compared with the quercetin Standard at 380 nm (a) Extract separated using Solvent I (Hexane/ Ethyl Acetate/ Chloroform/ Toluene - 81.52/4.07/9.97/5.43, v/v/v/v), (b) Extract separated using Solvent II (Toluene/ Ethyl Acetate/ Formic Acid - 54.35/43.48/2.17, v/v/v)

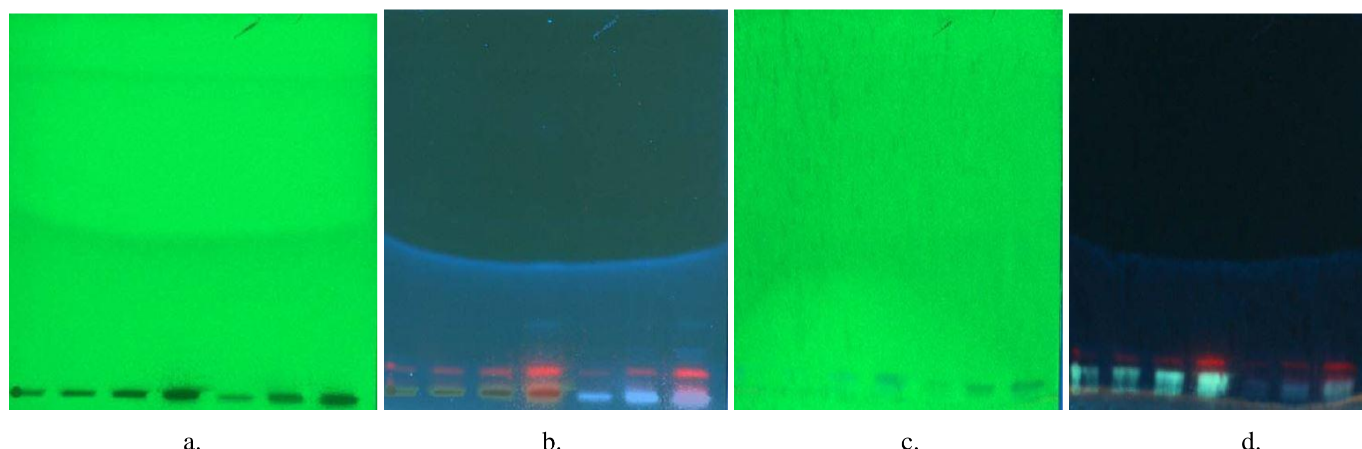


Fig 5: HPTLC chromatograms of the methanolic extract of *Elaeocarpus variabilis* seeds separated using Hexane/ Ethyl Acetate/ Chloroform/ Toluene (81.52/4.07/9.97/5.43, v/v/v/v) under Ultraviolet light. (a) Before derivatization viewed under 254 nm, (b) Before derivatization viewed under 366 nm, (c)After derivatization viewed under 254 nm, (b) After derivatization viewed under 366 nm

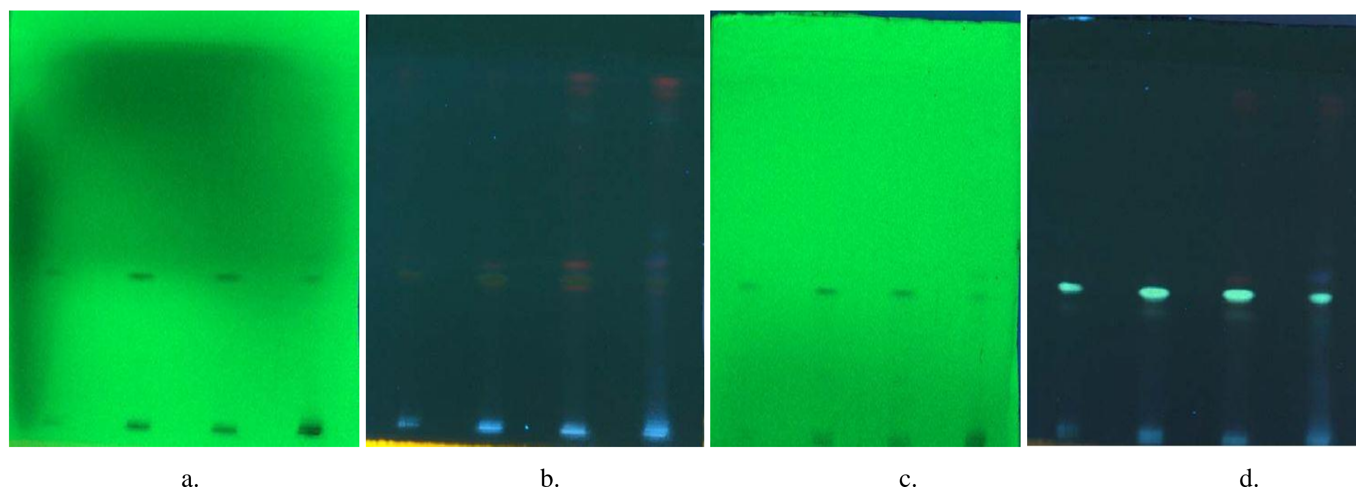


Fig 6: HPTLC chromatograms of the methanolic extract of *Elaeocarpus variabilis* seeds separated using Toluene/ Ethyl Acetate/ Formic Acid (54.35/43.48/2.17, v/v/v) under Ultraviolet light. (a) Before derivatization viewed under 254 nm, (b) Before derivatization viewed under 366 nm, (c) After derivatization viewed under 254 nm, (b) After derivatization viewed under 366 nm

Conclusion

The phytochemical profile for the identification and isolation of the active principle in the seeds of *Elaeocarpus variabilis* was qualitatively identified using chromatographic methods. From the previous studies, the method and solvents for were identified. Two solvents, methanol and acetone were used under cold percolation conditions which presented a yield (Yield %) of 9.4% and 2.4% respectively. Further, based on the results of qualitative analysis, the methanol extract of the seeds of *Elaeocarpus variabilis* were used for separation under Thin Layer Chromatographic. Optimization and standardization of TLC included different factors like thickness of the adsorbent, polarity of the compound, dissolution rate and mainly, identification of the optimum solvent system in the development of TLC. Finally, separation of compounds from the methanolic extract was done using the standardized solvent system - Hexane/ Ethyl Acetate/ Chloroform/ Toluene with the composition - 81.52/4.07/9.97/5.43 (v/v/v/v, in %) and the optimum thickness of the plate was identified to be 1mm. Further, TLC was scaled up to Preparative Thin Layer Chromatography and the purified extract was analyzedto quantify the desired compound using High Performance Thin Layer Chromatography (HPTLC). The results obtained from HPTLC after derivatization quantified the polyphenols and quercetin like compounds in the methanolic extract using two different developing systems. Further, the structural elucidation and quantitative estimation was performed using Liquid Chromatography couples Mass Spectrometric studies.

Acknowledgement

We are thankful to the Mr. P. Muthukumaran, Assistant Professor for his valuable inputs and grateful to the Head of Department, Department of Biotechnology and Principal, Kumaraguru College of Technology for providing facilities and encouragement.

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