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Research Article

CHROMATOGRAPHIC FINGER PRINTING AND QUANTIFICATION OF GALLIC ACID IN *AMLA* (*Emblica officinalis* Gaertn.), *PALASA* (*Butea monosperma* Taub.) AND *AMALAKI RASAYANA*

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ABSTRACT

Gallic acid (3, 4, 5-Trihydroxybenzoic acid) is a naturally occurring hydrolysable tannin and a potent antioxidant molecule. In Ayurveda, Rasayana therapy has been proposed as a remedy for ageing and associated ailments. The present study aims to analyse the gallic acid (GA) content in an important Rasayana used in Ayurveda viz., Amalaki Rasayana. High-Performance Thin-Layer Chromatography (HPTLC) method was carried out to delineate the phytoconstituents present in the methanolic extract of shade-dried powdered berries of Amla, the stem of Palasa and Amalaki Rasayana. The TLC plate developed in mobile phase toluene: ethyl acetate: formic acid (6:5:1v/v) had revealed 10, 6 and 5 phytoconstituents respectively for Palasa, Amla and Amalaki Rasayana. Densitometric scanning of GA had shown the absorption spectra λ max at 276 nm and polynomial regression analysis had revealed a good linearity response (r = 1.0000) in the concentration ranges of 1.44 – 15.84 µg/spot and mean content of GA in raw berries of Amla was estimated as 0.364% of its dry weight and that of processed Amla (i.e., Amalaki Rasayana) as 0.415%. The chromatographic data could fail to produce any detectable amount of GA in the stem of Palasa. The scaling up of the content of GA (5.33%) in thermal processing is suggestive that heat might have induced degradation of the polyphenols (viz., the polymers of ellagic acid, or gallic and ellagic acids) of the flesh of Amla berries, due to a combination of leaching, oxidation by polyphenol oxidase, enzymatic action and isomerization.

KEYWORDS: Amalaki Rasayana; HPTLC; Gallic acid; chromatogram; polyphenols

INTRODUCTION

Tannins are the major group of secondary metabolites found in plants and designated into two distinct chemical groups viz., the hydrolysable tannins and condensed tannins. Hydrolysable tannins are polymers of ellagic acid, or of gallic and ellagic acids with glucose whereas, the condensed tannins result from the condensation of monomers of flavan-3-ol units.

The high molecular weight tanniferous phenolic compound viz., gallotannins and ellagitannins on hydrolysis yield gallic acid and ellagic acid[1]. The first report on the synthesis of gallic acid from tannin was reported from the hydrolysis of Oak galls during the early 5thcentury and the name gallic acid was coined by Henri Braconnot in 1831. However, the biosynthetic pathway for gallic acid in plants still not yet fully elucidated. Gallic acid (Fig. 1) is a colourless crystalline phenolic compound of 3, 4, 5-Trihydroxybenzoic acid ($C_6H_2(OH)_3COOH$). Benzoic acid derivatives of phenolic (viz., vanillic acid, gallic acid, syringic acid) have a limited distribution in plants and are often considered as excellent antioxidants[2]. Their antioxidant capacity is attributed to the chemical structure of the hydrophobic benzenoid rings (inhibits the enzymes involved in the radical generation, such as several cytochrome P450 isoforms, cyclooxygenase, lipoxygenases and xanthine oxidase) and hydrogen-bonding potential of the hydroxyl groups. Hydrogen-donating electrons of these simple phenolics can react with reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the living tissues where these free radicals are stabilised by delocalisation which breaks up the cycle of generation of new radicals[3].

Being secondary plant metabolites, polyphenols are synthesized as defensive compounds against herbivory in a majority of fruits, herbs and vegetables. Polyphenol-rich diets and products have been linked to providing many benefits to humans. Gallic acid a simple phenolic compound obtained from the degradation of hydrolysable tannin has several commercial, pharmacological and therapeutic applications. Commercially gallic acid and its esters are being used for making paper, leather and as food additives[4]. Trimethoprim, an antibacterial agent, is produced from gallic acid and the polyphenols content of the analytes are estimated and often reported against the standard value of gallic acid equivalents. The pharmacological and therapeutic potentials of gallic acid have been explicated as antioxidant, antimicrobial, anti-inflammatory, and anticancer agents[5].

Kroes BH et al[6] have reported that Gallic acid has anti-inflammatory activity towards zymosan-induced acute food pad swelling in mice due to the interference with the functioning of polymorphonuclear leukocytes, scavenging of superoxide anions, and inhibition of myeloperoxidase releases. Intraperitoneal administration of gallic acid in hepatic reperfusion-induced rats has reduced hepatic cellular damage by the protection of sinusoidal endothelial cells[7]. It has neuroprotective activity[8]; and anxiolytic activity comparable to diazepam[9]. Gallic acid administered in streptozotocin-induced diabetic rats has pronounced antihyperglycaemic, anti-lipid peroxidative and antioxidant effects[10]. It has marked anti-Human Rhino Virus (HRV – 2 and HRV – 3) activity in human epitheloid carcinoma cervix (HeLa) cells[11]. Improved glucose tolerance and lipid metabolism by gallic acid in obese mice has been attributed to its anti-hyperglycemic activity by the upregulation of PPARγ

expression and Akt activation[12]. It also ameliorated carcinogenesis by activating ATM kinase signalling pathways[13]. Other conceivable reports demonstrating the role of gallic acid include prevention of gastric cancer cell metastasis, and invasive growth through expression of RhoB[14]; amelioration of tumor growth and progression in transgenic adenocarcinoma of the mouse prostate[15]; decreased glioma cell proliferation and tube formation in mouse brain endothelial cells, and prevention of human primary glioblastoma (U87) cell line invasion[16]. Gallic acid, both natural and synthetic analogue having protocatechuic acid alkyl esters, inhibits HIV – 1 protease[17]. The pro-oxidant and auto-oxidative behaviours of gallic acid have also been reported[18-19].

In Ayurveda, Rasayana therapy has been proposed as a remedy for Jara (i.e., ageing and ailments associated with it) and rejuvenation of the body[20]. There have been descriptions of numerous rasayana formulations in various treatises of Ayurveda with different indications comprising of plants, herbs, spices and sometimes minerals. However, the common aspect involved in all was that rasayana having the capacity to mitigate the ill-effects of ageing, either by delaying or reversing the process of ageing. In the above pretext, the scientific validations of the several of these Rasayanas are now under investigation by different laboratories and the available reports have revealed that the majority of such rasayana preparations are rich in antioxidant potentials capable of conferring immunomodulatory and anti-ageing effects[21-25]. Amalaki Rasayana is an important Rasayana formulation, which is being prepared (details of the preparation are described elsewhere) from the fruit of Amla (procured from the source plant *Emblica officinalis* (EO). The collected raw fruits of *Emblica officinalis* are then put into a specially created hollow cylindrical wooden vessel made of stem pieces taken from the *Butea monosperma* (Palasa) for thermal processing. Though the berries of Amla are a rich source of gallic acid, reports on estimating the content of Gallic acid in Amla are lacking in literature barring a few cases[26-29] and the content of gallic acid in Amalaki Rasayana has not been reported previously. Though there are findings of an increase in the gallic acid contents in a tune of about 8% to 152% in the boiled flesh of potato tuber, chestnuts and beans have been reported by other investigations, [30-32] the scaling up of gallic acid content in Amalaki Rasayana has not previously been reported.

In the above context, the present study was undertaken with the objectives of delineating the phytochemical profile of the stem of Palasa, raw fruits of Amla and Amalaki Rasayana. It also aims to estimate the content of gallic acid present in Amla and Amalaki Rasayana and in the stem of Palasa (in this study Palasa stem was used as a facilitator (i.e., wooden vessels) towards the thermal processing of the fruits of Amla for rasayana preparation). Further, the study aims to quantify the extent of gallic acid scale up in the flesh of Amla berries happening during the thermal processing.

MATERIALS AND METHODS

The materials used for the present study were collected from the plants growing in their natural habitats of the Paravur region of Kollam district of Kerala, India. The plants, *Butea monosperma* Taub. and *Emblica officinalis* Gaertn., were identified and authenticated by using Gambles flora of Presidency of Madras. Sufficient quantities of fresh berries of *Emblica officinalis* (EO) had been collected from the trees, and stem pieces from matured branch of *Butea monosperma* (BM) tree were used for making the wooden vessels for processing Amla fruits.

Preparation of Amalaki Rasayana

Three cylindrical stem pieces each having 25 cm length with bark had been removed from the straight medium sized BM branch. The central pith portions of the stem pieces were scrapped out so as to form a central hollow cavity of about 5 cm circumference and 23 cm length. The whole structure thus resembled as a vessel. Three circular lids were made using the same stem piece of palasa (BM) to enable the tight closure of the mouths of the vessels. Accurately weighed 240 g of freshly collected Amla fruits (EO) were filled into each of these BM wooden cylinders (Fig. 2) and the mouth of the vessels were closed by the prepared wooden lids. Then the surface of the vessels was smeared with mud in a specific way to attain a considerable thickness and heated slowly by increasing the temperature from 30 to 400^0C. The entire procedure adopted was as detailed in Ashtangahridaya[33]. After completion of the thermal curing, processed amla was taken out from the wooden vessels, and the seeds from each processed Amla fruits were carefully removed using a knife and flesh of the fruits were dried in the shade to achieve uniform weights. Likewise, to obtain uniform weights for the wooden vessels, stem pieces with a more or less the same thickness having intact bark was prune out from the mature branch of Palasa tree. Since then the wooden vessels were made available, the entire procedure for the preparation of rasayana, beginning right from the collection of fruits to the completion of thermal processing was finished within 48 hours duration.

The pulverized shade dried powder of Amla fruits, prepared Amalaki Rasayana (AR) and shade dried stem of BM (bark appressed with xylem portion only) were kept in separate, labelled, airtight glass containers for the further use for the preparation of TLC plates and densitometric scanning of the chromatograms (voucher samples are kept at the Department of R and B, Government Ayurveda College, Kannur with reference number 2018-06-03, 2018-06-04 and 2018-06-05 for EO, AR and BM respectively)

Development of Chromatogram by High-Performance Thin Layer Chromatography

Standard gallic acid (GA) used in the present study was procured from Avra Synthesis Pvt. Ltd, Hyderabad. HPLC/ chromatographic grade solvents viz., toluene, ethyl acetate and formic acid were purchased from Merck and Qualingens Fine Chemicals, India. The stationary phase used was the silica gel coated aluminium TLC plates 60 F_{254} (E. Merck KGaA, Germany) having the dimensions of 20 cm x 10 cm with a uniform thickness of 0.2 mm thickness.

Optimization of Chromatographic Conditions

Different combinations of the solvents with varying polarity were tried to achieve good chromatographic separation of the analytes on the TLC plates, and the most suitable mobile phase identified comprised of toluene: ethyl acetate: formic acid in the ratio 6:5:1, v/v.

Standard stock solution

14.4 mg of accurately weighed gallic acid (GA) standard was transferred to a volumetric standard flask and dissolved by adding HPTLC grade methanol, and the final volume was made up to 10 ml to form the stock solution. The concentration of gallic acid in working solution was 1.44µg/µl.

Sample preparation

For HPTLC densitometric analysis, two grams each of shade-dried powder of *Palasa* (BM) bark, berries of *Amla (*EO) and prepared Amalaki Rasayana (AR) were macerated with sufficient quantity of methanol for 48 hours with occasional shaking. The macerates were then filtered and the mark extracted twice in methanol. These three filtrates were then pooled and made up to a volume of 10 ml in a standard flask.

Instrumentation

The quantification of GA in the samples was carried out in CAMAG HPTLC system (CAMAG Switzerland) with Linomat V sample applicator having 100 µl syringe, CAMAG TLC viewer Reprostar, CAMAG Scanner 3 and winCATS Planar Chromatography Manager software (version 1.4.6.2002) and twin trough chamber of CAMAG Switzerland.

HPTLC Procedure

The stationary phase was activated by a hot hair dryer at a temperature of 60^0 C for five minutes, and the plate was placed in the Linomat V sample applicator. Six levels of working standard solution (1, 3, 5, 7, 9 and 11 µl) were applied on the TLC plate with a concentration ranges from 1.44µg to 15.84 µg (track 1 to 6) and sample solutions 4 µl each of BM, AR and EO were also applied on track number 6, 7 and 8. The bands were 6 mm in length. The plate was developed in a twin trough chamber (20 cm x 10 cm), which was pre-saturated by the mobile phase toluene: ethyl acetate: formic acid (6:5:1v/v); at a temperature of 25^0C and a relative humidity of 50 – 60%.

After completion of the development of the spots to 70 mm height, the TLC plate was observed in the TLC viewer at UV 254 nm, and photographs were taken. Densitometric scan of all peaks assignments was scanned (slit dimension of 6.00 X 0.45 mm) at UV 254 nm and 276 nm and the data on peak height, peak area and Rf values were recorded. The calibration graph was developed by plotting concentration against the peak area and the content of GA in EO, BM and EO samples were estimated by polynomial regression. The sensitivity of the HPTLC method was determined in signal-to-noise ratio of LOD (3Sa/b) and LOQ (Sa/b) as described by Srivastava et al[34] and Sudahakaran[35]. The experiment was done in triplicate, and statistical significance of the results was evaluated using t-test.

Table 1: Calibration data of gallic acid by polynomial regression method of HPTLC

Track	Identity	Vial	Rf	Amount fraction (µg)	Area	X(calc) (µg)
1	Standard 1	1	0.19	01.440	11631.36	
2	Standard 4	1	0.19	10.08	23462.98	
3	Standard 5	1	0.20	12.96	25340.73	
4	Standard 6	1	0.20	15.840	26250.73	
8	Sample AR	3	0.19		14988.16	3.233
9	Sample EO	4	0.19		14302.71	2.917

Table 2: Gallic acid content in raw berries of *E.officinalis* and prepared *Amalaki Rasayana*

Sample	Gallic acid content (µg)			Mean ± SD
EO	2.868	2.917	2.960	2.915 ± 0.04603
AR	3.323	3.280	3.358	3.320 ± 0.03907
P = 0.0003				

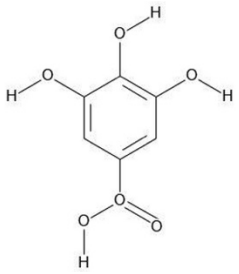

Figure 1: The structure of gallic acid

Figure 2: *Amalaki Rasayana* preparation: filling raw fruits of *amla* into wooden vessels of *Palasa*

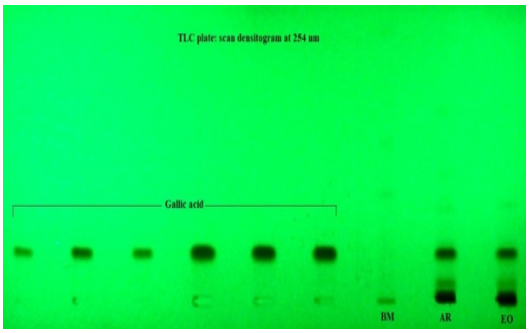

Figure 3: Scan-densitogram of methanol extracts of stem of *B.monosperma* (BM), *Amalaki Rasayana* (AR) and raw berries of *E.officinalis* (EO)

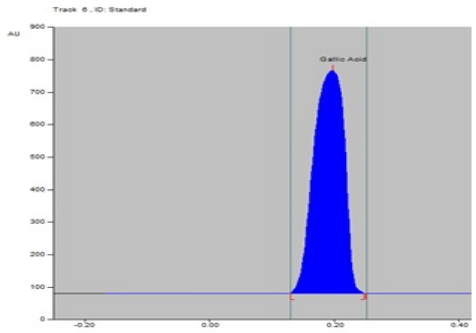

Figure 4: Densitogram of the standard gallic acid at 254 nm (15.84 µg/band) with *Rf* = 0.19

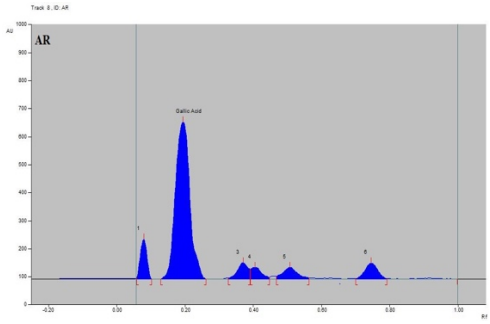

Figure 5: HPTLC fingerprint profile of methanol extract of *Amalaki Rasayana*

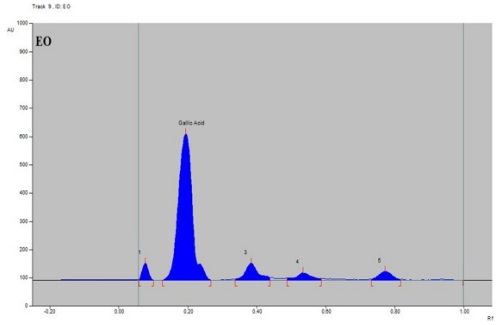

Figure 6: HPTLC fingerprint profile of methanol extract of berries of *E.officinalis*

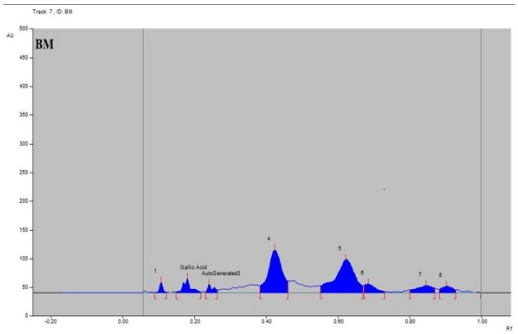

Figure 7: HPTLC fingerprint profile of methanol extract of stem of *B.monosperma*

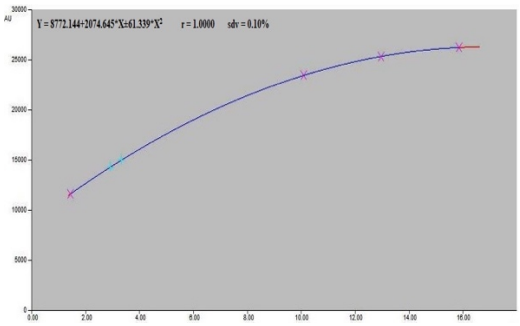

Figure 8: Calibration plot of gallic acid by polynomial regression (peak area vs concentration)

RESULTS AND DISCUSSION

HPTLC fingerprints of Amalaki Rasayana, *Emblica officianalis* and *Butea monosperma*

The chromatogram developed and the resolved bands obtained were depicted in Fig. 3. In the figure track no. 1, 2, 3, 4, 5, and 6 denote the bands for standard gallic acids and track no. 7, 8, and 9 for bands obtained for methanol extracts of the stem of Palasa (BM), prepared Amalaki Rasayana and berries of Amla (EO) samples respectively. The developed chromatogram under UV 254 nm had revealed a good separation of GA with Rf value of 0.19 in the standards as well as in the samples (Fig. 4, 5 and 6). The methanol extract of AR (track no.8) and EO (track no. 9) showed strong, distinctive spots for GA, whereas track no. 7 contained methanol extract of BM failed to produce any pronounced band relative to GA at Rf 0.19 (Fig. 7), suggesting that BM didn't contain any detectable amount of GA. Densitometric scanning of GA had shown the absorption spectra at λ max 276 nm. The overlay spectra of standard against the extract of BM at 276 nm could also fail to produce any spectral matching, further confirming the absence of any spectroscopically detectable amount of GA in the stem of *Butea monosperma*.

Linearity and Calibration curve

Evaluation of linearity for GA was performed via peak area vs concentration. The concentration ranges used for plotting the calibration curve were 1.44 – 15.84 µg/spot. The calibration curve obtained was found to be linear (Fig. 8) in the concentration ranges from 1.44 to 15.84 µg/spot with a correlation coefficient r = 1.0000 and a standard deviation of 0.10%. Analysis of calibration equation ($Y = 8772.144+2074.645*X\pm61.339*X^2$) had shown that the mean content of GA in a raw sample of amla was found to be 0.788µg/µl and Amalaki Rasayana as 0.83µg/µl (Table. 1) and these differences (Table. 2) were found to be statistically significant (P = 0.0003). Further, it could also estimate the content of GA in raw berries of Amla as 0.364% of its dry weight, and that of processed amla (i.e. Amalaki Rasayana) was 0.415%. The scaling up of gallic acid content obtained in Amalaki Rasayana is suggestive that the thermal processing of

Amla berries in wooden vessels of BM, the heat might have induced degradation of polyphenols of the flesh of Amla berries, due to a combination of leaching, oxidation by polyphenol oxidase, enzymatic action and isomerisation. Consonant findings of an increase in the gallic acid contents in a tune of about 8% to 152% in the boiled flesh of potato tuber, chestnuts and beans have been reported by other investigations.

The sensitivity of the HPTLC method

The estimated values for the limit of detection (LOD) and limit of quantification (LOQ) for the present study were calculated 0.159ng and 0.482ng respectively from the regression equation have shown the sensitivity of the developed method. The developed HPTLC method is simple, easy to apply in quality control parameters for the standardisation of berries of amla and Amalaki Rasayana and also useful for the detection of gallic acid and its quantification in the herbal formulations/ayurvedic drugs/natural products.

CONCLUSION

Ayurveda uses a variety of dosage forms prepared through different processing methods from various raw materials of herbal, mineral and animal origin for application in the preservation of health as well as the mitigation of ailments. These methods of drug processing and preparation are primarily intended for reducing the toxicity of the raw materials, increasing the therapeutic potential of the finished drug and improving the palatability of dosage forms. Here we have demonstrated that thermal processing of fruits of *Emblica officianalis* inside the wooden vessels of *Butea monosperma* stem has significantly increased the gallic acid content in the finished product, the Amalaki Rasayana. Throughout the world, 80 per cent or more of the population relies on Ayurveda like traditional medicines for their primary health needs, and therefore it is essential to ensure the quality of Ayurvedic products reaching the market. Marker compound based chromatographic procedures have been demonstrated as reliable methods for quality control measures of herbal medicine and Ayurvedic drugs in regulatory perspectives. The chromatographic fingerprinting developed by HPTLC method in the present study by using Gallic acid as a marker compound can be considered as a reliable method for assessing the genuineness berries of amla in herbal formulation and finished products.

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