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Identification and characterization of Ethyl gallate from Ethyl acetate fraction of Phyllanthus emblica fruit, And invitro free radical scavenging activity

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Abstract*---*Phyllanthus emblica fruit has a rich source of polyphenolic compounds and is widely used due to its medicinal properties. A portion of the ethyl acetate fraction (EAF) from 70% methanol extract (70% ME) showed greater 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activities. Moreover, EAF has more polyphenol content than 70% ME. The EAF was subjected to a dry silica gel column eluted successively with a mixture of chloroform: ethyl acetate: formic acid (2.8: 2.4: 0.4ml) as a mobile phase solvent system, and to afford various fractions. EAF and isolated fraction-1 of ethyl gallate (EG) showed a unique pattern of EG standard chromatogram in thin layer chromatography (TLC) analysis. EAF and isolated fraction-1 real time (R.T) of the peaks were unique compared with those of the standard chromatogram in high-performance liquid chromatography (HPLC) analysis. This chromatogram fraction was

collected from isolated fraction-1 at the same R.T time by multiple HPLC analysis. The collected fraction from HPLC was injected into Mass spectroscopy (MS) and it showed a molecular ion at m/z 197 corresponding to $C_9H_{10}O_5$. The substance was identified as EG based on the results mentioned above.

***Keywords*---**Phyllanthus emblica, radical scavenging activity, antioxidant, polyphenols.

Introduction

Phyllanthus emblica also known as Emblica officinalis is commonly known as Indian gooseberry or Amla belonging to the Euphorbiaceae family. Amla is an essential herb used in the Ayurvedic and Unani medical systems. (Muthuraman et al., 2011). It is found in India, China, Uzbekistan, Malaysia, Pakistan, Sri Lanka and South East Asia. Amla is a highly nutritious fruit that is abundant in amino acids, minerals and vitamin C. It has a number of different chemical components, including tannins (punigluconin, pedunculagin, emblicanin A and emblicanin B), terpenes (lupeol, gibberellin A-1, gibberellin A-3, gibberellin A-4, and gibberellin A-9), flavonoids (quercetin-3-O-ß- D-glucoside and kaempherol-3-O-ß-D-glucoside); sterols (ß-daucosterol) and phenolics (ellagic acid and gallic acid). (Dhingra et al., 2012). Ethyl gallate (EG) is a known compound in E. officinalis (Yang et al., 2007) and it is an additive for food with the E. number E313. The EG structure has been shown in figure 1. According to studies, EG exhibits antiparasitic (Calderon et al., 2006), anticancer (Yoshioka et al., 2000: Saleem et al., 2002), antimicrobial (Shibata et al., 2005), protective effect of Acute lung injury (Mehla et al., 2013) and radical scavenger (Zheng et al., 2009) activities. Recently, EG identified in Aonla pasteurized juice extract (Kumari et al., 2019). However, the objective of the current study is to separate and characterise ethyl gallate from the ethyl acetate (EtOAc) fraction of dried E. officinalis fruits. Additionally, evaluate and compare the DPPH and ABTS scavenging capacities and total polyphenol content in 70 ME and EAF.

Material and Methods

Plant Material and chemicals

The dried fruit of *Phyllanthus emblica* (Phyllanthaceae) were procured locally in Hyderabad, India and authenticated by Professor H. Ramakrishna of the department of Botany, Osmania University, Hyderabad, and Telangana, India. It was identified and voucher specimen deposited at the (no. 322) Botany Department of S.V. University, Tirupathi, A.P, India.

Chemicals

EG, DPPH and ABTS were purchased from Sigma–Aldrich, USA. Methanol, hexane, chloroform and ethyl acetate were purchased from SD fine chemicals for fruit powder extraction. Acetonitrile, formic acid and water were purchased on LC-MS grade for analysis of extraction.

Extraction and isolation

The amla fruit extraction procedure has been shown in Figure 2. Briefly, the dried fruits were grounded to fine powder using electronic grinder, and 100g of Amla was extracted with 70% methanol (70% ME) at room temperature (500 ml) with constant stirring on magnetic stirrer for 1 hour, after the extract was filtered through Whatman filter paper (110 mm) for a clear solvent, the resultant solvent was rota evaporated and dried completely and the weight of the total 70% ME amla extract was 34g. From the yielded extract, 30g was taken and re-dissolved in 750ml of double distilled water followed by extracted with an equal volume of hexane, chloroform and ethyl acetate. Part of the EAF solvent was rota evaporated and dried completely and the weight of the total EAF extract was 1.826g.

Silica gel column chromatography

A portion of the EAF was placed to a dry silica gel column eluted successively with mixture of chloroform: ethyl acetate: formic acid (2.8: 2.4: 0.4ml) as a mobile phase solvent system, and to afford various fractions. Therefore, isolated fraction-1 was further subjected to thin layer chromatography (TLC) and hplc chromatography.

Thin layer chromatography (TLC)

A portion of the ethyl acetate solvent fraction and isolated fraction-1 were subjected to analytical TLC over silica gel plate (TLC silicagel 60 F254) using a mixture of chloroform: ethyl acetate: formic acid (2.8: 2.4: 0.4ml) as a mobile phase solvent system. The spots were located by exposing the plate to iodine fumes.

HPLC analysis

The HPLC analysis of EAF from 70% ME *E.officinalis* dried fruit and isolated fraction-1 from Part of the EAF and EG standard ware performed using Shimadzu Sil-HTC model, Separations were performed on an Agilent SB-C18 (4.6×150 mm, 1.8 µm) column using the following method. (A) 0.2% formic acid in water and (B) acetonitrile are the mobile phases. Here, a gradient solvent system was used; 0-3 min, 5% B; 3-9 min, 25% B; 9-17 min, 37% B; 17-17.20 min 5% B and 17.20-21 min, 5% B; With monitoring at 272 nm, the flow rate was 0.2 ml/min. At a temperature of 20°C in the oven, the injection volume was 20 µL. The parts of the EAF and isolated fraction-1 were subjected to HPLC chromatography and the real time (R.T) of the peaks in the sample chromatograms was compared with that of the standard chromatogram to identify the compound, and EG fraction was collected from isolated fraction-1 at the same R.T time by multiple HPLC analysis.

MS analysis

A method of analysis called mass spectrometry (MS) determines the mass-to-charge ratio of charged particles. It is used to calculate particle masses, identify molecules, and reveal the chemical composition of molecules including peptides,

polyphenols, and other chemical compounds. The 4000-QTRAP triple-quadruple hybrid mass spectrometer (Applied Biosystems, Foster City, CA, USA) was used to identify phenolic compounds such as EG from the EAF of amla extract. This was done with ESI-MS analysis.

DPPH Assay for determining in vitro free radical scavenging/antioxidant potential assays

70% ME and EAF were tested in triplicate for their capacity to scavenge free radicals in DPPH (Peterson et al., 2001). Briefly, 25 µl of the extract at 5 mg/ml in DMSO was added to 100 µl of tris buffer (0.1 M, pH 7.2) and 125 µl of a 0.5 mM methanolic DPPH solution. In order to calculate the mixture's absorbance at 517 nm, it was incubated for 30 minutes at room temperature and in the dark. Free radical scavenging activity of the DPPH was demonstrated by the IC50 value, which is the concentration of sample needed to cut the absorbance at 517 nm by 50%.

ABTS Assay for determining in vitro free radical scavenging/antioxidant potential assays

In accordance with the published procedure, the ABTS free radical scavenging activity of 70% ME and EAF of extracts were assessed in triplicate (Re et al., 1999). In a nutshell, the reaction between 2.45 mM potassium persulfate and 7 mM ABTS created the ABTS+ radical cation. The reaction mixture was used two days after being left to remain at room temperature for 16 hours in the dark. Ethanol was used to dilute the ABTS+ solution, yielding an absorbance of 0.70 ± 0.050 at 734 nm. A volume of 10 µl of 70% ME and EAF (5 mg/ml in DMSO) were added individually to 190 µl of the ABTS radical solution. Before measuring the absorbance at 734 nm, the mixture was kept for 15 minutes at room temperature and in the dark. The ABTS+ scavenger rate was used to numerically express the 70% ME and EAF extract's antioxidant capability. The IC_{50} values depend on the concentration needed to lower the absorbance at 734 nm by 50%, which is represented by the calculated IC_{50} values.

Total polyphenol content (TPC)

1 mg/ml of sample extract was taken and 5 ml of Folin-Ciocalteu reagent was added and mixed thoroughly using vortex. 4 ml of Na_2CO_3 solution was added and was incubated for 60 mins at room temperature. The absorbance was read at 765 nm in a spectrophotometer. TPC is expressed as gallic acid equivalents in mg/100g material (Tachakittirungrod et al., 2007).

Results and Discussions

Results
Thin layer chromatography (TLC)

EAF and EG, isolated fraction-1 and EG standard were analyzed in TLC; EAF and isolated fraction-1 of EG showed with unique pattern of EG standard chromatogram (Fig. 3a & 3b). This indicate EG present in *E.officinalis* fruit.

HPLC and MS

Quantitative analysis of EAF extract of *E.officinalis* and isolated fraction-1 were performed on Shimadzu Sil-HTC model equipped with a Photo Diode Array (PDA) detector system following the procedure, briefly chromatographic analysis were performed on an Agilent SB-C18 (4.6×150 mm, 1.8 µm) column, utilizing a mobile phase consisting of (A) 0.2% formic acid in water (B) acetonitrile. A gradient solvent system was used; 0-3 min, 5% B; 3-9 min, 25% B; 9-17 min, 37% B; 17-17.20 min 5% B and 17.20-21 min, 5% B; the flow rate was 0.2 ml/min with monitoring at 272 nm. The injection volume was 20 µL at 20˚C oven temperature. Standard EG solutions were put into the system, and the chromatograms were taken at 19.31 real time, which was the same as the standard conditions that had been mentioned before. (Fig. 4). Under the same chromatographic conditions, the EAF and isolated fraction-1 were prepared and injected into the system. The Real time (R.T) of the peaks in the EAF (19.318) and isolated fraction-1 (19.307) chromatograms are compared with that of standard chromatogram to identify the compound as EG (Fig. 5a & 5b) and EG fraction was collected from isolated fraction-1 at same R.T time by multiple HPLC analysis. This EG fraction injected into MS for conformation of molecular weight and compared with that of EG standard molecular weight to identify the compound as EG (Fig. 6).

Amla extracts were evaluated for antioxidant potency by using the DPPH assay

In this study, 70% ME and ECF of DPPH scavenging screening were done at a concentration of 0.2 mg/mL and by taking Vitamin C (0.2 mg/mL) and Rutin (0.2 mg/mL) as reference standards. The maximum inhibition of DPPH by EAF was 87.94% greater than that of 70% ME (Table). The IC50 values of 70% ME and EAF were 113 µg/ml and 50 µg/ml respectively, while those of ascorbic acid and rutin were 13 µg/ml and 16 µg/ml respectively (Table 1).

Amla extracts were evaluated for antioxidant potency by using the ABTS assay

In the present study, 70% ME and EAF (EAF) of ABTS radical scavenging screening were done at a concentration of 0.2 mg/mL and by taking Vitamin C (0.2 mg/mL) and Rutin (0.2 mg/mL) as reference standards. The maximum inhibition of ABTS radical by EAF was 97.39% greater than that of 70% ME, which was a little less effective than that of standard. The IC50 values of 70% ME and ECF were 17 µg/ml and 5 µg/ml respectively, while those of ascorbic acid and rutin were 4 µg/ml and 3 µg/ml respectively (Table 1).

Determine the total polyphenol content on Amla extracts

The polyphenol content in 70% ME and EAF was found to be 37.973 ± 0.96 mg/100g and 59.537 ± 0.96 mg/100g (n=3).

Discussion

The fruits of *E.officinalis* collected from local market and extracted with 70% ME, extracts *evaporated under vacuum.* In the current study, to obtain the hexane fraction, chloroform fraction, ethyl acetate fraction, and aqueous fraction, the methanol extract was further partitioned in water with hexane to defat the extract, then chloroform was used to remove highly nonpolar chemicals that might stick to the column, and finally the extract was extracted with ethyl acetate. (*Mahajan & Pai, 2010).* Ethyl acetate layer evaporated under reduced pressure on rotary evaporator to yield 1.826g of Ethyl acetate fraction. In this study we isolated *EG* from ethyl acetate fraction. Part of the EAF was applied to a dry silica gel column eluted successively with mixture of chloroform: ethyl acetate: formic acid (2.8: 2.4: 0.4ml) as a mobile phase solvent system, and to afford various fractions. *The TLC chromatogram data of* EAF *and isolated fraction-1* of EG showed with unique pattern of EG standard chromatogram.

EAF and isolated fraction-1 were performed on HPLC. When compared to a standard chromatogram, the Real Time (R.T.) of the peaks in the sample chromatograms is unique. This chromatogram fraction was collected from *isolated fraction-1* at same R.T time by multiple HPLC analysis. The collected fraction from HPLC was injected into MS and it showed a molecular ion at m/z 197 corresponding to $C_9H_{10}O_5$ (Zhang et al., 2009). From the above results, it is concluded that the compound is EG.

Using a standard spectrophotometric test, the DPPH scavenging abilities of 70% ME extract and EAF were assessed. EAF showed the greatest DPPH scavenging activity than 70% ME. The antioxidant activity of natural substances can also be calculated using their reactivity with ABTS radicals (Miller et al., 1996). In the present study, ABTS assay of EAF noticeably showed free radical scavenging potential than 70% ME extract. The findings of prior investigations are consistent with the results of our study. (Chaphalkar et al., 2017). According to previous reports, the amount of polyphenols in this fruit has also been linked to a lot of antioxidant activity, especially when it comes to DPPH and ABTS, which are two types of free radicals. Moreover, biological effects of amla polyphenols showed cardio-protective, anti-diabetic, anti-cancer, anti-inflammatory, and neuroprotective activity (Gul et al., 2022).

Conclusion

The expected chemical constituent was identified in the EAF from the fruit of a 70% methanol E. officinalis extraction and also isolated. The isolated compound was confirmed as EG. EAF showed more antioxidant potential by DPPH and ABTS scavenging than 70% ME. Apart from this, EAF has more polyphenol content than 70% ME. Ethyl gallate is also one of the active constituents in the amla fruit, which possesses more antioxidant potential activity to scavenge free radical stress in human health disorders. Based on our investigation, we suggest that EG could be useful for patients suffering from oxidative stress disorders.

Acknowledgments

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Conflict of Interests

The authors declare no conflict of interests.

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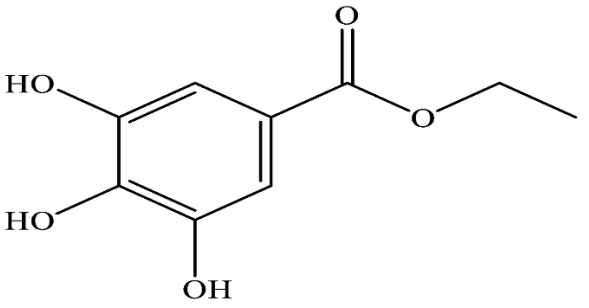

Fig. 1. Chemical structure of ethyl gallate.

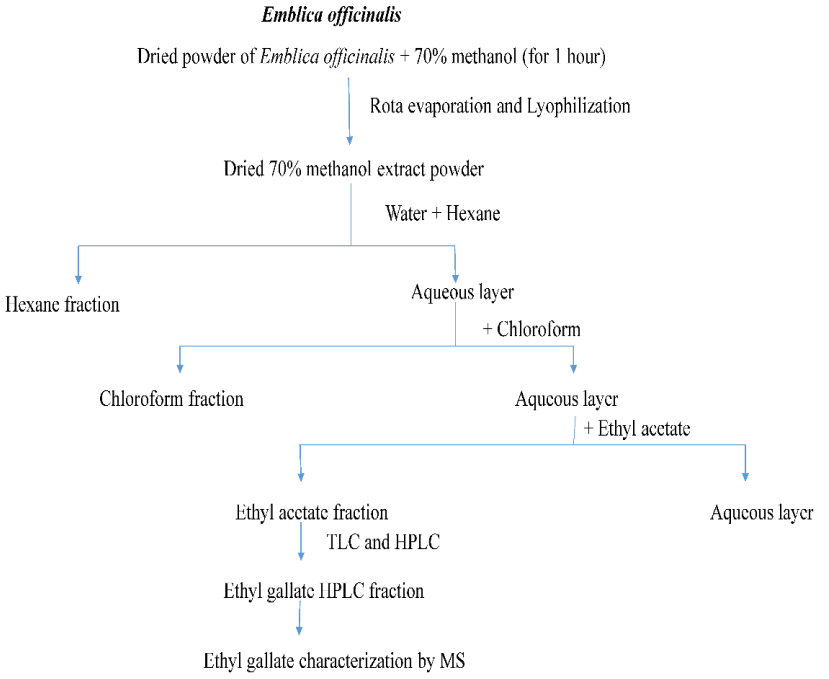

Figure 2 Schematic diagram for the identification and characterization of ethyl gallate from *E.officinalis* Linn.

Figure 3 Thin layer chromatographic pattern of 70% methanol *E.officinalis* extract by maceration method (3a) and ethyl acetate fraction (3b). Chloroform: ethyl acetate: formic acid (2.8: 2.4: 0.4ml) used as a mobile phase solvent system. TLC plate exposed to iodine fumes. S1 and A1 are indicate ethyl gallate standards and, T1 is 70% methanol extract and B1 is ethyl acetate fraction.

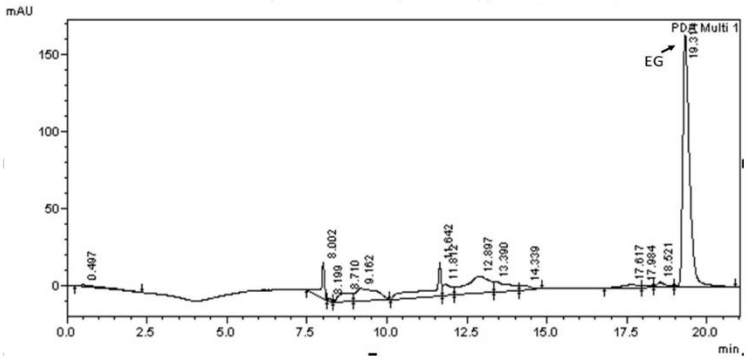

Figure 4 High-performance liquid chromatography (HPLC) analysis of ethyl gallate standard using a SB-C18 column.

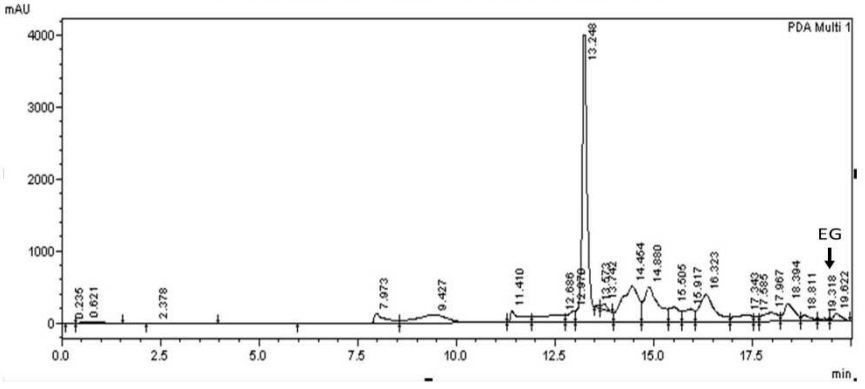

Figure 5a

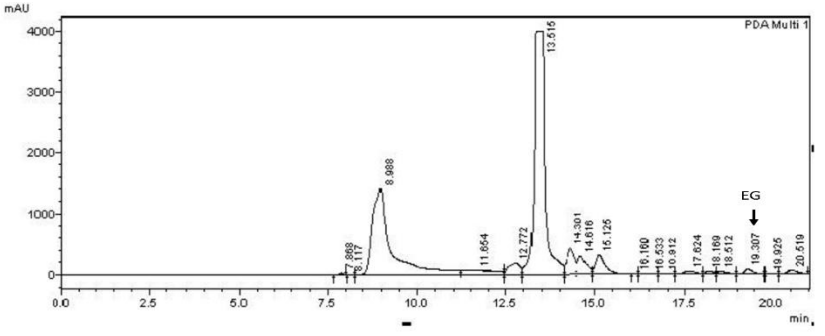

Figure 5b

Figure 5 HPLC analysis of ethyl acetate fraction of *E.officinalis* (4a) and isolated fraction-1 (4b) using a SB-C18 column. HPLC chromatogram showing crude compounds resolved at their respective retention times (RTs).

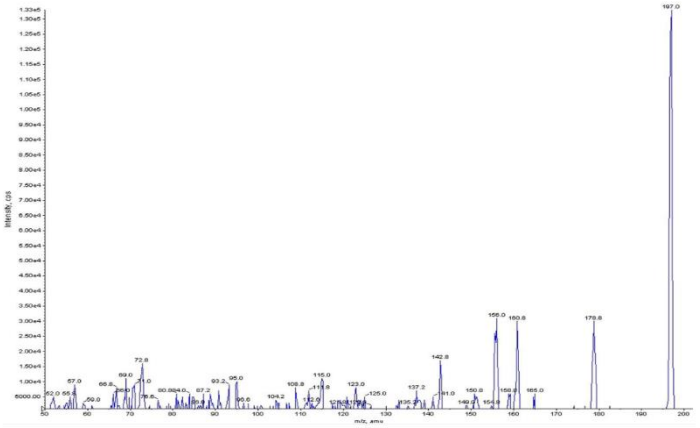

Figure 6 MS analysis of isolated fraction from ethyl acetate fraction.

S.No	Assays	70% ME	EAF	Standard Vitamin-C	Standard Rutin
Percentage inhibition (0.2 mg/ml)	DPPH	82.24%	87.94%	90.05%	81.84%
	ABTS	96.90%	97.39%	100%	99.01%
IC50 (mg/ml)	DPPH	0.113±0.001	0.050±0.001	0.013±0.001	0.016±0.001
	ABTS	0.017±0.001	0.005±0.0001	0.004±0.0001	0.003±0.0001

Table 1: The DPPH and ABTS scavenging activities of 70% ME and EAF of *E.officinalis*.