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GC-MS Analysis, Antioxidant and Free Radical Scavenging Activity of *Emblica Officinalis* Gaertn (Amla) Fruit Extracts

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ABSTRACT
Emblica officinalis (also known as *Phyllanthus emblica*) belongs to the family Euphorbiaceae, is commonly known as "Amla" and widely distributed in the tropical and subtropical areas, particularly in central and southern India, Pakistan, Bangladesh, Srilanka, Southern China and Malaysia. This plant shows a variety of biological activities such as anti-inflammatory, antipyretic, diuretic, and laxative, anticancer, antioxidants and anti-diabetes etc. Therefore, the aim of the present study is to evaluate phytoconstituents, antioxidant potential, total phenolic & flavonoid contents of *E. officinalis* fruit extracts. Further, TLC and GC-MS analysis was determined. Phytochemical analysis confirmed the presence of alkaloids, phenol, tannin, saponin, glycoside, terpenoids, flavonoids, carbohydrates, amino acids, and protein in the Aqueous and Methanolic Extracts of *Emblica officinalis* fruit. In TLC total 06 spots were present in the methanolic extract with different Rf values. Total phenolic content was found to be 274 ± 29.58 mg GAE/g, flavonoids content was 130.2 ± 21.58 mg QE/g. Whereas antioxidant activity was dose dependent. Many biologically active components were present as analysed by GCMS. The highest peak area of Oleic Acid (3.325%) was observed.
Keywords: *E. officinalis,* Ascorbic acid, Free radicals, Antioxidant Activity, GC-MS, Total Phenolic content, Total Flavonoid Content

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INTRODUCTION

Natural Medicinal plants and herbal drugs represent a major allocation of all the recognized systems of health in the world. Traditionally medicinal plants have been used for the ailments of different countries for ancient periods [1]. *Emblica officinalis* Gaertn. (Family–Euphorbiaceae) also known as *Phyllanthus emblica*, belongs to Euphorbiaceae family and commonly known as "Amla" or "Amlaki"in Bengali and "Indian gooseberry" in English [1]. Since Ancient times, *Emblica offcinalis* have been used as a "rejuvenating herb" in Ayurveda [2].From various researches it reveals that "Amla" is known for its medicinal and nutritional properties [3]. Many herbal and patent drugs have been formulated by the constituents of this plant [4]. E. offcinalis is widely distributed in the tropical and subtropical areas, particularly in central and southern India, Pakistan, Bangladesh, Srilanka, Southern China and Malaysia [1, 3]. The fruit is also used in a combination form known as Triphala meaning three fruits which is a Thai traditional herbal formulation composed of *Emblica officinalis, Termi-nalia belerica* and *Terminalia chebula* [5]. *Emblica offcinalis* contains tennis, flavonoids, phenolic compounds, saponins, derpenoids, ascorbic acid, carbohydrates and many other compounds [6]. Supplements of fresh amla fruit is very favorable to individuals suffering from anemia. The juice of fresh amla fruit is given as diuretic, anti-bilious remedy and as a tonic. It is also helpful in over thirst, dyspepsia, burning sensation and other complaints of digestive system [7]. Plants show a variety of biological activities, ie as anti-inflammatory, antipyretic, diuretic, and laxative,[8] anticancer,[9] antioxidants, anti-diabetes [10-11]. Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) are often generated as byproducts of biological reactions or from exogenous factors.[12] These reactive species exert oxidative damaging effects by reacting with nearly every molecules found in living cells [13]. Free radicals are reported to be the major cause of some serious pathogenic disorders, such as cardiovascular diseases, atherosclerosis, neurodegenerative disorders, diabetes, cancer, liver cirrhosis, inflammation and cataracts [14]. Due to the presence of the conjugated ring structures with hydroxyl groups, many phenolic compounds by scavenging superoxide anion [15], singlet oxygen [16], lipid peroxy radicals [17] and stabilizing free radicals involved

in oxidative processes through hydrogenation or complexing with oxidizing species have the potential to function as antioxidants [18]. Antioxidants are one of the components needed in the body, to counteract free radicals. Excessive free radicals in the human body can cause several diseases, such as diabetes, heart disease and inflammation [19]. The antioxidant compounds obtained from plants may be phenolic, carotenoids [20-21] compounds, and flavonoids [22]. Methanolic extract of fruit pulp also have antioxidant and free radical scavenging activity [23-27]. Galic acid equivalent as total phenolic content from fruit and seed of *E. officinalis* has excellent antioxidant proper-ties and play an important role as free radical scavengers required in the maintenance of, redox homeostasis responsible for diverse degenerative diseases [28]. Therefore the aim of this study was to investigate the phytoconstituents, antioxidant Capacity, total phenolic and flavonoids content of the methanolic extracts of the *E. officinalis* fruits.

MATERIALS AND METHODS

Collection of Plant Material

E. officinalis fruits were collected from the local market of Jhansi District. All the fruits were washed to remove dust particles with tap water followed by de-ionized water for two to three times. Fruits separated from the seeds and chopped into small pieces and kept in dark for drying at room temperature. Dried chopped pieces crushed and stored in air tight jars for extraction purpose.

Extraction Procedure

Crushed fruit of *E. officinalis* was extracted by Aqueous as well as Methanolic extraction method.

Aqueous Extract

Different concentration 5 gm and 10 gm of prepared powder of *E. officinalis* chopped fruit was extracted with same volume (100 ml) of de-ionized water in conical flasks. Flasks were heated in the water bath at 90°C and left for 1 hour. After completing 1 hour extracts were filtered and stored at 4°C.

Methanolic Extract

30 gm of *E. officinalis* fruits powder was extracted through the soxhlet apparatus at 60-65°C by using the 300 ml of 80% methanol as a solvent. Repetition of the cycle was continued until the solvent appeared colurless. Filtered extract was evaporated at 40 ^{0}C and stored at 4 ^{0}C.

Screening of Phytochemicals

The screening was carried out with some modifications [29-31], for the presence and absence of the secondary metabolites.

Total Phenolic Content (TPC)

The total soluble phenolic content present in methanolic extract of *E. officinalis* fruits was estimated by using the protocol of Folin- Ciocalteu method [32]. 100 µl of various concentrations from 1000 to 62.5 µg/ml were mixed with adding the 500 µl of double distilled water. In these all tubes mix the 100 µl of Folin-Ciocalteu reagent and allowed to stand for 6 minutes. To this mixture add the1 ml of 7% sodium carbonate and adjust the volume by adding the 500 µl of double distilled water. All of these mixture tubes were then incubated in dark at room temperature for 90 minutes. The absorbance was measured against blank at 760 nm by using multi plate reader. As plotting the standard curve, gallic acid was used. The total phenolic content was showed as mg of gallic acid equivalents (mg GAE/g).

Total Flavonoid Content (TFC)

The total soluble flavonoids content of the methanolic extract of *E. officinalis* fruits was done by the method of Piyanete *et al.* [33]. Flavonoid content was expressed as dry weight of quercetin equivalent (mg QE/g). 100 µl different concentration of methanolic extract and same volume of the quercetin dilution was mixed with 500 µl of distilled water followed by 100 µl of 5% sodium nitrate and allowed to stand for 6 minutes. To this mixture 150 µl of 10% Aluminium chloride solution was added and incubated at room temperature for 5 minutes, and finally complete the assay by adding 200 µl solution of 1M Sodium hydroxide. The absorbance of this reaction mixture was recorded at 510 nm.

Thin layer chromatography

Based on *in vitro* results, TLC plates coated with silica gel-G of 0.2 mm thickness was used for the testing of methanolic extract of *E. officinalis* fruits. Here we used a solvent mixture (Butanol- acetic acid-water) at the ratio of 2:1:1 v/v as described by somewhere else. Spotting the methanolic extract above 4mm from the base of the plate, spots migrate with the solvent mixture on the silica coated plates by the capillary action. Fully developed silica coated plate was air dried followed by heating for 20-25 minutes. The plate was sprayed with 0.2% freshly prepared ninhydrin solution to detect the bands.

These spots were expressed by its retention factor (Rf).

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Total antioxidant capacity (TAC) by Phosphomolybdenum assay

According to Prieto et al [34] phosphomolybdenum assay was used for the total antioxidant capacity of the methanolic extract of *E. officinalis* fruits. 0.2 mL of different concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) of the extract was combined with 2 mL of reagent solution. Same concentration was used for standard ascorbic acid and 80% methanol was used for a blank tube. All the tubes of sample, blank and standard with reaction mixture were incubated for 90 min at 95°C. Followed by cooling at room temperature and measured the absorbance at 695nm. Calibration curve was drawn with the respect of ascorbic acid.

Nitric oxide radical scavenging assay

Free radicals generated from sodium nitroprusside (SNP) were measured according to the earlier described method [34] with some modifications. Different concentration of reaction mixture containing SNP (15 mM) in PBS (pH 7.3) with and without sample, incubated at 25°C for 210 mins. Add Griess reagent followed by rest for 10 minutes at room temperature. Ascorbic acid was used as a standard. The absorbance was measured at 560 nm using a UV-Vis microplate reader.

Superoxide anion scavenging assay

The total antioxidant capacity of methanolic extract was based on the reduction of NBT according to a previously reported method [35] with some modification. The 1-mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), PMS (60µM), NBT (156µM), and various concentrations of sample solution. After incubation for 5 min at 25°C temperature, the absorbance was taken at 560 nm against an appropriate blank solution. BHT was used as positive control.

(GC-MS) Analysis

On a Perkin Elmer Turbo Mass Spectrophotometer with a Perkin Elmer autosampler XLGC, GC-MS analysis of methanolic extract of *E. officinalis* was performed. The column was a Perkin Elmer Elite - 5 capillary column with a film thickness of 0.25 mm and a length of 30 m. It was made of 95 percent dimethylpolysiloxane. At a flow rate of 0.5 ml/min, carrier gas helium (99.999 percent) was used as the carrier gas. As an injection length, a 1 l sample was used. The inlet temperature of the GC was maintained at 250 °C, with a programmed oven temperature of 110 °C (isothermal for 2 min), followed by a 10 °C/min increase to 200 °C, followed by a 5 °C/min increase to 280 °C, and a 5 °C/min increase to 280 °C, with a 5 °C/min isothermal at 280 °C. The GC took 30 minutes to run. The temperature of the MS transfer line is kept at 200°C, while the source temperature is kept at 180°C. The GCMS analysis was carried out using electron impact ionisation at 70eV, and Total Ion Count was used for data 60 Sharma and Kumar evaluation of compound detection and quantification (TIC). The spectrum of the components was compared to the known components stored in the GC-MS library. For peak area measurement and data processing, Turbo-Mass OCPTVS-Demo SPL programme 19 was used.

RESULTS

For the presence of various secondary metabolites, screening of both aqueous and methanolic extract of *E. officinalis* fruits shows in the (Table 1). Used solvent is responsible for the both presence and absence of these secondary metabolites and methods are also responsible which applied for their qualitative detection. Both aqueous and methanolic extract of amla fruits shown positive results except Mayer's test for aqueous extract. For the detection of carbohydrates Molish's shows all the positive results where as Barfoed's tests shown negative results. Fehling's and Benedict test was used for the detection of reducing sugar, which shown all the positive results either in aqueous or methanolic extract. Both test of Flavonoids, alkaline reagent and lead acetate tests shown positive results except alkaline reagent test for 10 gm of aqueous extract. All the tests for glycosides had shown mostly positive results except Borntrager's shown negative in aqueous and Keller-killiani in methanolic extract. Different tests of Tannin, Phenolic and hydrolysable tannin showed all the positive results. Like tannin saponin was also present in both extract. Whereas amino acids and proteins are totally absent in both extracts. The thin layer chromatography of methanolic extract of *E. officinalis* fruits shows the presence of total 06 spots with their unique Rf values 0.85, 0.79, 0.61, 0.55, 0.41 & 0.35 respectively (Fig. 1). Here we used alanine amino acid as a standard and its Rf value was 0.69. The percent inhibition of methanolic extract was found to be 88.53% at a concentration of 1000 mg/ml whereas ascorbic acid, on the other hand, had a scavenging activity of 90.8% at the same concentration (Fig. 2). As a result, the antioxidant potential is due to the phenolic and flavonoid material. The mean values of total phenolic and flavonoid contents were 274.8 ± 29.58 mg GAE/g &130.2 ± 21.58 mg QE/g respectively (Table 2). The nitric oxide and superoxide radical scavenging behaviour of the methanolic extract of *E. officinalis* was dose dependent (Fig.3 and 4). The active principle analyzed by GCMS with molecular weight, molecular formula, peak area %, retention time, and composition of the bioactive components of methanolic extract of *E. officinalis* fruits (Table 3). The GC-MS chromatogram of the

identified compounds is shown in (Fig. 5). Many biologically active components were observed. The highest peak area of Oleic Acid (3.325%), 9-Octadecenoic acid (Z), octadecyl ester (3.060%), Hematoporphyrin (2.056%), psi.,.psi.-Carotene, 3,4-didehydro-1,2,7',8'-tetrahydro-1-methoxy-2-oxo (2.025%), 2-Dodecenal (1.248%), Perylo[1,12-def]-1,3-dioxepin-5,11-dione, 6,12-dihydroxy-8,9-bis(2- hydroxypropyl)-7,10-dimethoxy-, stereoisomer (1.201%), 2,4-Bis(5-chlorothien-2-yl)-6-dicyanomethylene-2-methyl-1,2,3,6-tetrahydopyridin-5-carbonitrile (1.030%), 2-Tridecenal (0.993%), (E)-, Delsoline (0.981%), Lutein (0.916%), Rhodopin (0.903%) and Phorbol 12,13-dihexanoate (0.857%) shown different biological activities like Antioxidant, anticancerous, antimalerial and antileishmanial activities etc.

DISCUSSION

Biological studies of plant extracts have been carried out to verify the pharmacological properties of the plants. The radical scavenging, reducing capacity and metal chelating properties of antioxidants are known to eliminate and prevent the generation of free radical [36]. The properties have been contributing directly or indirectly in the prevention of pathogenesis and deterioration of food, [37-38] whereas the ability of plant extract to kill or inhibit the growth of microorganisms is at interest for the development of antimicrobial agent. Thus, such studies add value and provide scientific information to continually validate the potential of the plant known as ethnomedicine.[38-39] Free radicals are reported to be the major cause of some serious pathogenic disorders, such as cardiovascular diseases, atherosclerosis, neurodegenerative disorders, diabetes, cancer, liver cirrhosis, inflammation and cataracts [40]. Due to the presence of the conjugated ring structures with hydroxyl groups, many phenolic compounds by scavenging superoxide anion [41], singlet oxygen [42], and lipid peroxy radicals [43], and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species have the potential to function as antioxidants [44]. Amla fruit is a rich natural source of vitamin C (70-72%), 20 times as much as present in orange juice and tannin. Due to rich vitamin C, amla is successfully used in the treatment of human scurvy. Fruit has also been reported to contain phyllemblic acid (6.3%), gallic acid (5%), lipid (6%), emblicol, flavaniod, colloidal complexes and micic acid. Phyllembin, from fruit pulp identified as ethyl gallate potentiate the pharmacologic action of adrenaline *in vitro* and *in vivo* [45].

Table 1: Qualitative phytochemical analysis of the aqueous and methanolicextracts of *E. officinalis* fruits

S No.	Phytochemical Test	Aqueous Extract		Methanolic Extract
		10 gm	5 gm	
1.	**Alkaloids** Mayer's Wagner's Hager's	- ve + ve + ve	- ve + ve + ve	+ ve + ve + ve
2.	**Carbohydrates** Molisch Barfoed's	-ve -ve	+ ve - ve	+ ve - ve
3.	**Reducing Sugars** Fehling's Benedict's	+ ve + ve	+ ve + ve	+ ve + ve
4.	**Flavonoids** Alkaline ReagentLead Acetate	- ve + ve	+ ve + ve	+ ve + ve
5.	**Glycosides** Borntrager's Legal's Keller-killiani	- ve + ve + ve	- ve + ve + ve	+ ve + ve - ve
6.	**Tannin & phenolic**Ferric Chloride Lead Acetate Dilute iodine solution	+ ve + ve + ve	+ ve + ve + ve	+ ve + ve + ve
7.	**Saponin** Froth	+ ve	+ ve	+ ve
8.	**Protein & A.A.** NinhydrinBiuret	- ve - ve	-ve -ve	-ve -ve
9.	**Triterpenoids &Steroids** Salkowski's	+ ve	- ve	+ ve
10.	**Hydrolysable tannin**	+ ve	+ ve	+ ve

(+) indicates presence while, (–) indicates the absence of the components

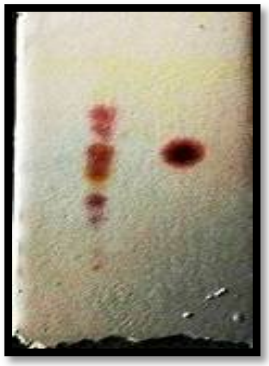

Figure 1. TLC plate showing spots having different Rf values (0.85, 0.79, 0.61, 0.55, 0.41 & 0.35) of methanolic extract of *E. officinalis* fruits

Table 2. Total Flavonoid & Phenolic Content of methanolic extract of *E. officinalis* fruits

Conc. of extract (µg/ml)	Total Flavonoid Content (mgQE/g)	Conc. of extract (µg/ml)	Total Phenolic Content (mgGAE/g)
1000	85	150	289
500	126	120	304
250	96	90	304
125	136	60	158
62.5	208	30	319
Mean ± SEM	**130.2 ± 21.58**	**274.8 ± 29.58**	

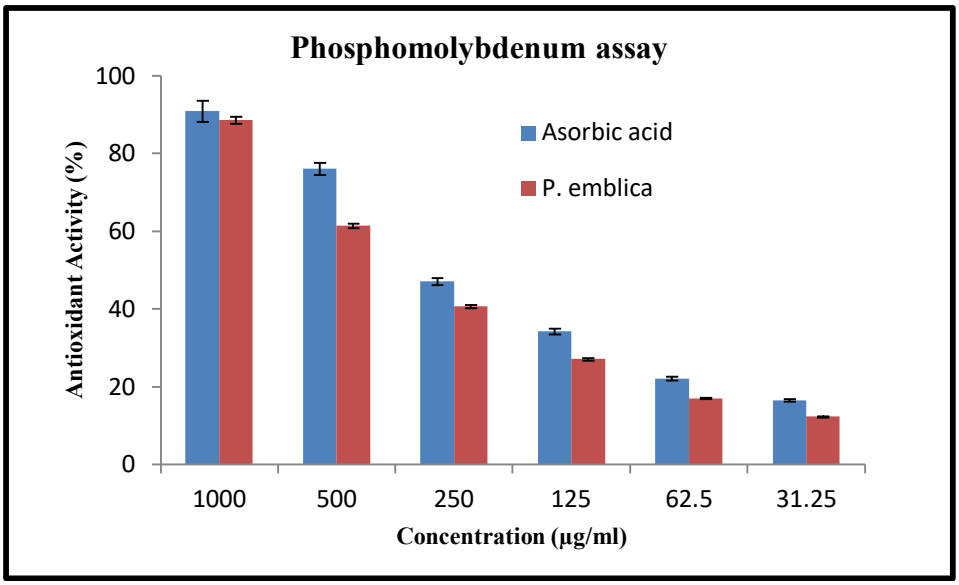

Figure 2: The Antioxidant activity of methanolic extract of *E. officinalis* fruits

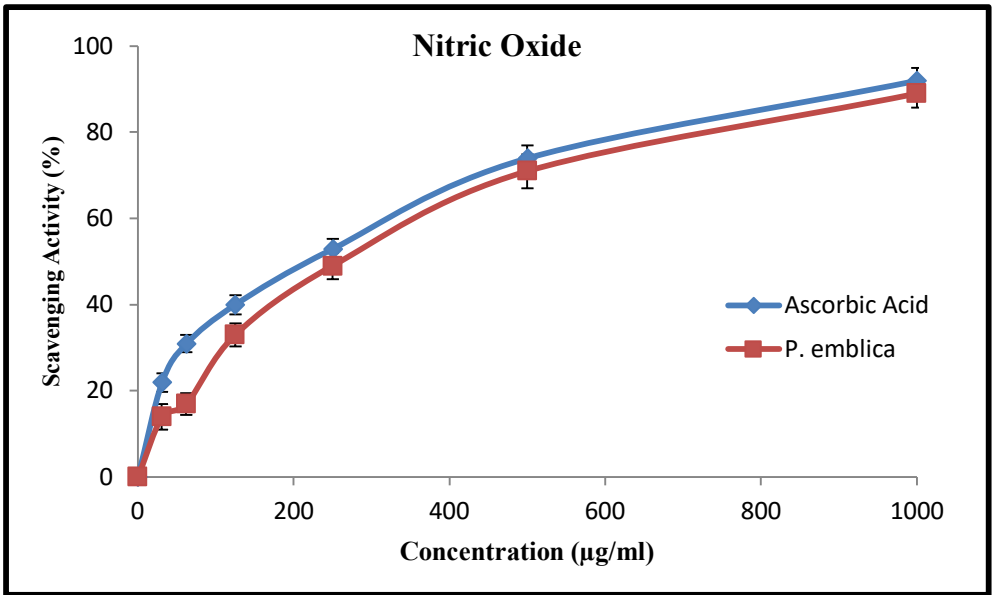

Figure 3: Nitric oxide scavenging activity of methanolic extract of *E. officinalis* fruits

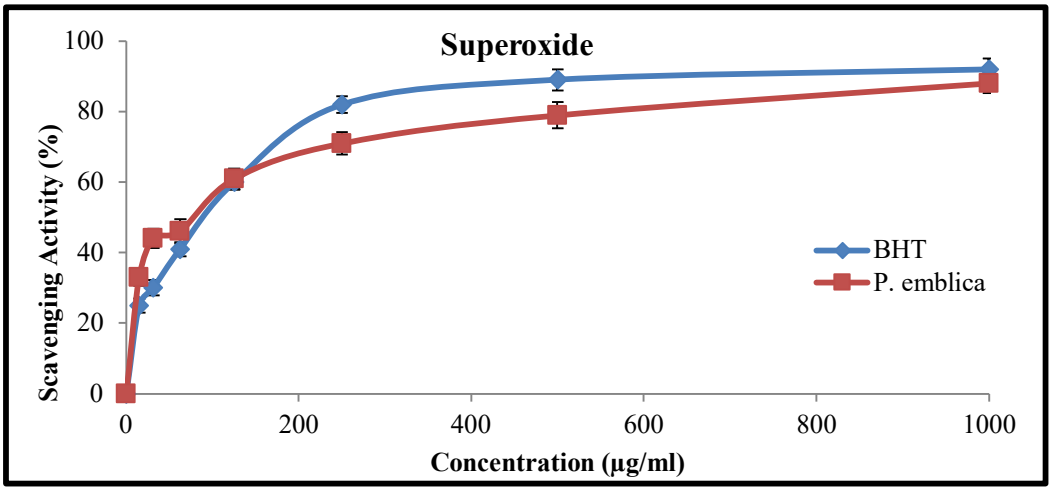

Figure 4: The superoxide radical scavenging activity of methanolic extract of *E. officinalis* fruits

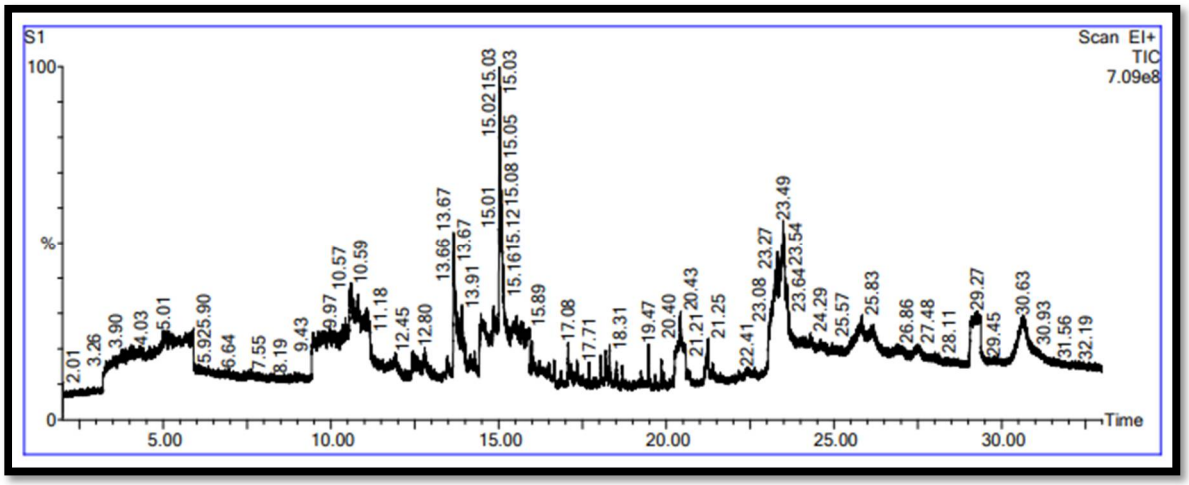

Figure 5: Total Ion Chromatogram of methanolic extract of *E. officinalis* fruits

Table 3: Compounds identified by GC-MS analysis of methanolic extract of *E. officinalis* fruits

Sr. No.	RT	PP %	Compound name	M.F.	M.W.	Biological Activities
1.	3.414	0.857	Phorbol 12,13-dihexanoate	$C_{32}H_{48}O_8$	560	Antiviral activity 46
2.	10.441	0.903	Rhodopin	$C_{40}H_{58}O$	554	Bacterial metabolite 47
3.	5.354	0.916	Lutein	$C_{40}H_{56}O_2$	568	An antioxidant Plant metabolite 48
4.	9.477	0.981	Delsoline	$C_{25}H_{41}NO_7$	467	Anti-malarial 49
5.	13.673	0.993	2-Tridecenal, (E)-	$C_{13}H_{24}O$	196	NCI Yeast Anticancer Drug Screen. Data for the rad50 strain, for the bub3 strain Nematicidal activity against Bursaphelenchus xylophilus at 0.5 mg/ml measured after 48 hr under microscope 50
6.	9.972	1.030	2,4-Bis(5-chlorothien-2-yl)-6-dicyanomethylene-2-methyl-1,2,3,6-tetrahydopyridin-5-carbonitrile	$C_{18}H_{10}Cl_2N_4S_2$	417	NCI human tumor cell line growth inhibition assay. Data for the OVCAR-5, OVCAR-3 , OVCAR-8 Ovarian cell line, RPMI-8226 Leukemia cell line, SR Leukemia cell linenci-H23 Non-Small Cell Lung cell line 51
7.	11.069	1.201	Perylo[1,12-def]-1,3-dioxepin-5,11-dione, 6,12-dihydroxy-8,9-bis(2-hydroxypropyl)-7,10-dimethoxy-, stereoisomer	$C_{29}H_{26}O_{10}$	534	Antimalarial activity Antileishmanial activity Antimalarial activity 52
8.	15.031	1.248	2-Dodecenal	$C_{12}H_{22}O$	182	An anthelminthic drug, a plant metabolite and an antibacterial agent Nematicidal activity against bursaphelenchus 53
9.	5.007	2.025	.psi.,.psi.-Carotene, 3,4-didehydro-1,2,7',8'-tetrahydro-1-methoxy-2-oxo-	$C_{41}H_{58}O_2$	582	Bacterial metabolite 54
10.	5.664	2.056	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	598	Phototherapy of malignant neoplasms 55
11.	23.317	3.060	9-Octadecenoic acid (Z)-, octadecyl ester	$C_{36}H_{70}O_2$	534	NCI In Vivo Anticancer Drug Screen. Data for tumor model L1210 Leukemia in B6D2F1 (BDF1) mice 56
12.	23.492	3.325	Oleic Acid	$C_{18}H_{34}O_2$	282	An EC 3.1.1.1 (carboxylesterase) inhibitor, An Escherichia coli metabolite, a plant metabolite, a Daphnia galeata metabolite, a solvent, anantioxidant and a mouse metabolite 57

**** Biological Activities Based on the PubChem Database**

CONCLUSION

Study of different medicinal plants shows different types of economical and curative use of medicines. This study concluded that *E. officinalis* fruit is a potential Source for natural antioxidants. Antioxidant activity of this study is dose dependent and for this activity the presence of phenols and flavonoids are responsible. There are different types of secondary metabolites present like alkaloids, reducing sugar, flavonoids, glycosides, tannins, phenolic compounds, saponins. But the presence of these secondary metabolites was varying in different solvents. Overall, the methanolic extract of amla fruit was the most effective solvent for phytochemical studies. According to presence of vitamins, minerals and other compounds, there is no doubt to say that it is a versatile medicinal plant.

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CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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