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# Separation and Identification of Many Natural Products from *Emblicaofficinalis* Fruits and Study of their Antibacterial Activity

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***Abstract*****—** *The active constituents were separated from Emblicaofficinalis fruit, using column chromatography (CC) with solvent system of (pet.ether:ethyl acetate; 5:1) from petroleum ether extract ($A_1$) and solvent system (chloroform:Methanol; 10:1) from Ethanol extract ($A_2$). Saponification process was carried out on ($A_1F_2$) to separate many fatty acids and identification of five fatty acids (Heptanoic, Octanoic, Lauric, Palmitic and also cis& trans Oleic acids) using GLC-analysis. Moreover, acid hydrolysis was also carried out on ($A_2F_2$), to separate many free poles of phenolic compounds, such as (Hydroquinone and Gallic acid) were identified using HPLC-analysis. The active separated compounds under study showed different effects against the micro-organisms (m.o), using disc diffusion method turbidity.*

***Keywords*****— *GLC analysis, Emblicaofficinalis, fatty acid, phenolic compounds.***

## I. INTRODUCTION

*Emblicaofficinalis* (amla) belongs to the familyEuphorbiaceae. It is one of the most often used herb in Ayurveda. It has a reputation as a powerful rejuvenating herb. Amla is a medium-sized deciduous tree with gray bark and reddish wood. It is native to tropical southeastern Asia, particularly in central and southern India, Pakistan, Bangladesh and Sri Lanka. It is commonly cultivated in gardens throughout India and grown commercially as a medicinal fruit. Amla fruit is reputed to have the highest content of vitamin C. the major chemical constituents of amla are phyllemblin, ascorbic acid (vitamin C), gallic acid, tannins, pectin (Priya et al., 2012).Amla is hepatoprotective, anti tumor, antioxidant, antiulcerogenic and antimicrobial against a wide range of bacteria (Sairam et al., 2002; Rajkumar et al., 2014).Amla has been found rich in unsaturated acids. Most predominant polyunsaturated fatty acids like (Lauric, Myristic, Palmitic, Stearic, Oleic, Linolenic and Linoleic and its rich in phenolic compounds like Ascorbic acid, Gallic acid, Chlorogenicacid, Ellagic acid, Myricetin, Quercetin and Kaempferol (Arora et al., 2011; Bansal et al., 2014).The antimicrobial properties of methanolic seed extract of amla was evaluated against various microbial *E. coli*, *Staph. aureus*, *K. pneumoniae* and *P. aeruginosa*. The sensitivity test for the bacterial species on the methanolicseed extract of amla showed exhibited good activity as similar results (Priya et al., 2012).

## II. MATERIALS AND METHODS

Amla fruits were collected from local markets of Mosul city then classified and verified by Dr. TalalTaha(a director of the medicinal plants project in Mosul Dam project) according tothe sources of classification.

**Preparation of plant extracts:**A batch of 50gm of the ground fruits of amla was Soxhlet extracted for 6-8 hrs. with 1L of two solvents (petroleum-ether and ethanol). All these extracts were concentrated to 25 ml on a vacuum rotary evaporator at 50°C. the crude of each extract was used for further studies.

**Fractionation of petroleum-ether and ethanoliccrude extract:** 300 mg of each crude extract which was previously prepared was mixed with a small amount of Silica gel 25 gm and then transferred to the top of a prepared Silica gel (60-120 mesh) column. The column was eluted with (pet-ether-ethyl acetate; 5:1) V/V intervals. Dissolved 1 gm of each extract of petroleum ether $A_1$and ethanolic $A_2$ on the basis of amla fruits. Separation of the extract wasperformed using a Silica gel filled column type Silica gel 60 A for flash chromalography filled with 30 gm of gels with petroleum ether-ethylacetateas a solvent (5:1 V/V) and code for P.E for crude petroleum ether extract, two fractions were obtained $A_1F_2$, $A_1F_3$. The ethanol extract has been filled with solvent chloroform: Methanol

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(10:1 V/V) Ch:M, several fractions were obtained $A_1F_2$ depending on the similarity of the spots in their positions and shapes on the TLC plate. And for the same solution system used in the column. The parts were evaporated by rotary evaporator RVE under pressure.

**Saponification of the extracts:** Saponification process was carried out using petroleum ether extract which belongs to ($A_1$) from (petroleum ether: ethyl acetate) (5:1) to gel $A_1F_2$ and $A_1F_3$ as a resulted from column chromatography. Moreover, the fraction of $A_1F_2$ was also saponified to librate free fatty acids, also acid hydrolysis was done. Also, the crude ethanolic extract was fractionated to (chloroform-methanol) (10:1).The fraction $A_2F_2$was subjected to saponification to while the $A_2$ fraction were acid hydrolysed.

**Alkaline hydrolysis to obtain fatty acid compounds:** Pet-ether extracts and the fractions of petroleumether:ethylacetate resulted from column chromatography.A mixture of 10 gm of each pet-ether extract and also the fractions (petroleumether:Ethylacetate) from petroleum ether extracts ($A_1F_2$) and 100 ml of 7-5 M of solution of KOH in MeOH:water (3-2) was refluxed in a round bottom flask for 20 min at 100°C. The suspension crude extract was allowed to cool down at room temperature, and 100 ml of distilled water was added. The crude was extracted with diethyl ether (2×100 ml) to remove unhydrolysed1ipid. The hydrolyte was acidified with 20% (V/V) conc. $H_2SO_4$ up to PH=2. the librated fatty acids were extracted with diethyl ether (2×100 ml) (Aruther, 1972).The combined extracts were washed with water and dried over anhydrous sodium sulfate, filtered and concentrated in vacuum to give 1.2 gm of crude fatty acid compounds.

**Preparation of Methyl Esters:** 0.1 ml of acetyl chloride was added to 25 ml dry methanol with stirring,. Also, the sample of 0.5 ml of dry fatty acids was added to the above mixture, then boiled under reflux in a water bath for 20 min and dry closed system, then cooled for analysis by GLC method (Al-Kaisy, 1991).

**Acid hydrolysis to separate free pole phenolic compounds:** The fractions of (Chloroform:methanol) (10:1) resulted from column chromatography. A mixture of 5 ml of fraction (Chloroform: methanol) from ethanol extracts $A_2F_2$ and 25 ml of (1N) HCl was refluxed in around bottom flask for 1 hr. at 100°C. after cooling, the solution was transferred to the separating funnel then (2×5 ml) of ethyl acetate was added with stirring and after isolation of organic layer from aqueous layer, the organic layer was dried by adding 10 gm of magnesium sulfate for 10 min. The ethyl acetate was concentrated under vacuum rotary evaporates and then kept the free pole of phenolic compounds in glass bottles for further analysis (Harborne, 1998).

**GLC-Analysis:** The fatty acid methyl esters can be obtained either directly by transmethylation of the parent lipids by refluxing for 90 min-with (MeOH-benzene $H_2SO_4$) (20:10:1) or from the free acids by the acetyl Chloride-Methanol. The esters were analysed by using GLC on a packurd 419 equipped with adanl flame ionization detectors at 270°C temp. A(2-12 m×2m) international diameter column. Packed with 3% silar10°C on Gas Chrom Q (100-120m) was initially, then raised to 5°C for one min to 20. The identification of fatty acidswere determined by references to a standard of known composition (Bauer et al., 1966).

**HPLC-Analysis:** The free pole of phenolic compounds was identified by using HPLC apparatus with shimadzo-2010 AHT after purification by filters with a diameter of 0-1 micrometer, and using 320 nm wavelength and 1 ml/min flow rate and (85/ Acetonitrile:15. $H_2O$) as a mobile phase by using the column of C18 (4×240mm) at30°C temperature. The instrument was provided from a Cherey company Naged and all measurements were carried out in the laboratory of University of Mosul.

**Antibacterial activities of prepared extracts of amla:** The antibacterial effect of these extracts were tested on all studied bacteria according to the method of modified Kirby-Bauer (Adam, 2006) in which the inoculums were prepared in nutrient broth and incubation at 37°C for (18-24) hrs. The density of the tested suspensions was adjusted to 0.5 McFarland Standard Muller-Hinton agar plated was inoculated by dipping a sterile cotton swabs in the inoculums the excess inoculums were removed by pressing and rotating the swabs firmly against the side of the tubes above the level of the liquids and the swabs were streaked all over the liquids and the swabs were streaked all over the surface of the solution.

Finally, the inoculums were left to dry for few minutes later 50 ml of each of the prepared extracts were dried and re-dissolved in DMSO and placed in walls 6 mm in diameter which were placed on the inoculated plates, the antibiotic discs were also placed. The plates were incubated at 37°C for (18-24) hrs. the diameter of each zone of inhibition (including the diameter of wells and discs) were measured, recorded and compared with the standard antibiotics (Gentamicin (CN) 10 meg, Amikacin (AK) 10 meg, ciprofloxacin (10 meg). Also we have used various concentration (200-50) mg/cm$^2$ of plant extracts under study (Asif, M., 2011).

## III. RESULTS AND DISCUSSION

During the phytochemical screening and previous studies (Priya et al., 2012; Sairam et al., 2002; Rajkumar et al., 2014) of the amla composition which were mentioned with many fatty acid compounds and many phenolic compounds which were investigated in the fruits of amla. From the study of above we have noticed and identified many fatty acid

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compounds, by using GLC-analysis. Table 1. and Figure 1. and Figure2. showed the presence of five fatty acid compounds within the fraction $A_1F_2$they are Heptanoic (0.0079%), Octanoic (0.0138%), Lauric (0.0161%), Palmitic(0.045%) and (cis& trans)Oleic acid (0.172%).

In this study, major unsaturated fatty acids were found such asOleic acid in this species, at high percentage (Priya et al., 2012). Oleic acid was also found as a major fatty acid which play an important role in the body as it is combined with omega-3 to many health benefits (Okpako and Ajibesin, 2015). Also two phenolic compounds were identified Table 2. and Figure 3. and Figure 4. in the fruit of amla. The fraction $A_2F_2$ which were found the column as acid hydrolysis took place to release free phenolic compounds to get HPLC analysis which is explaining as follows. The fraction $A_2F_2$ was contained Gallic acid with $R_t$(2.048 min) and Hydroquinone $R_t$(2.537 min).The results were similar to previous studies(Bansal et al., 2014; Agarwal et al., 2012). Table 3. and Table 4. and Photos 1-3. showing the antibacterial activity of amla (fatty acids & phenolic extract) against some pathogens bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*).

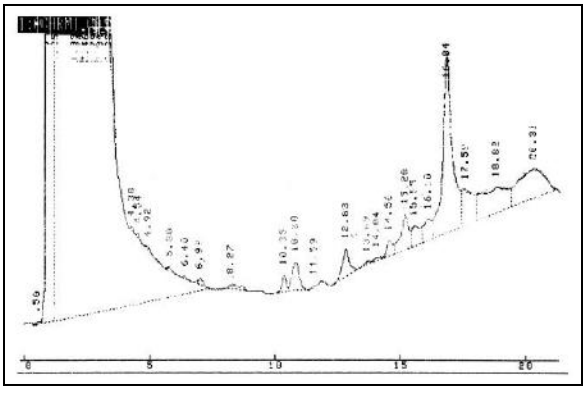
The results showed that fatty acid extracts ($A_1F_2$) and phenolic compound ($A_2F_2$) at (200 mg/c$^3$) per disc possessed high antibacterial activity against *Staph. aureus* and *Escherichia coli* whereas it was moderately active against *Salmonella typhimurium* compared with antibiotic compounds (Gentamicin (CN) 10 meg, Amikacin (AK) 10 meg, and Ciprofloxacin (Cip) 10 meg). From previous studies (Nitiema et al., 2012) we focused on some phenolic compounds action against bacteria involved in cute gastroenteritis diarrhea.

The study (Priya et al., 2012) results by using seeds of amlathat it contain high antibacterial and antioxidant activities and can be further studiedfor the isolation of its bioactive compound. So, the amlaand Cayratiapedata exhibited a potential of in-vitro antimicrobial activity against *Helicobacter Pylori* which suggest that it may be useful for the treatment of *H. Pylori* infection (Rajkumar et al., 2014).

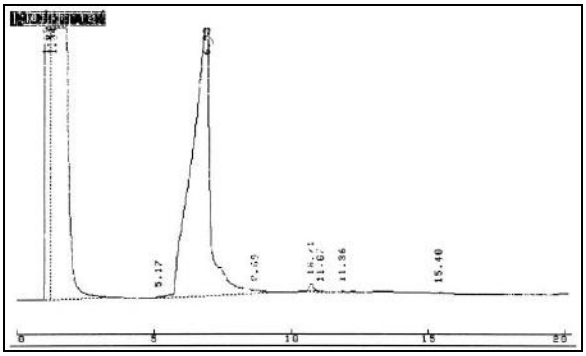
Thus, several studies have reported the antimicrobial activities of plant extracts from various parts like leaves, seeds and flowers (Karon et al., 2005; Falleh et al., 2008). These results often pointed out that crude extract possesses low antibacterial activities against enteric bacteria (Karon et al., 2005; Zongo et al., 2011).

*Table.1: GLC analysis of amla extracts of fatty acid compounds.*

| Extracts of amla | Fatty acid compounds | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Heptanoic | | Octanoic | | Lauric | | Palmitic | | cis& trans Oleic acid | |
| | $R_t$(min) | Conc. | $R_t$(min) | Conc. | $R_t$(min) | Conc. | $R_t$(min) | Conc. | $R_t$(min) | Conc. |
| Fraction $A_1F_2$ | 6.999 | 0.0079 | 8.273 | 0.0138 | 12.835 | 0.0161 | 15.558 | 0.0451 | 18.825 | 0.172 |



Fraction $A_1F_2$



Heptanoic

*Fig.1: GLC chromatograms of fatty acid compounds presented in amla.*

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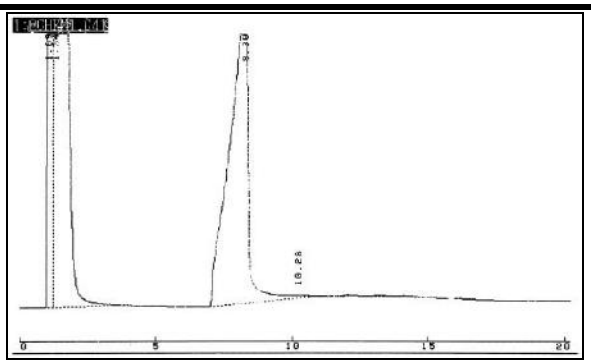
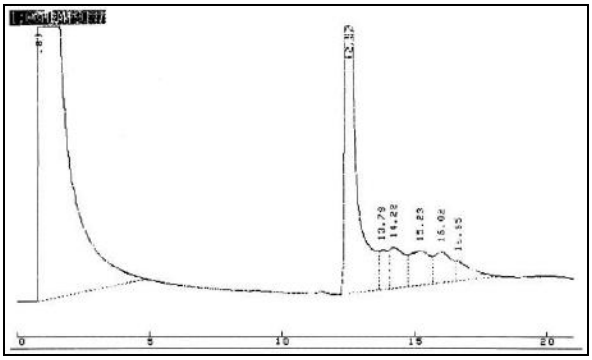
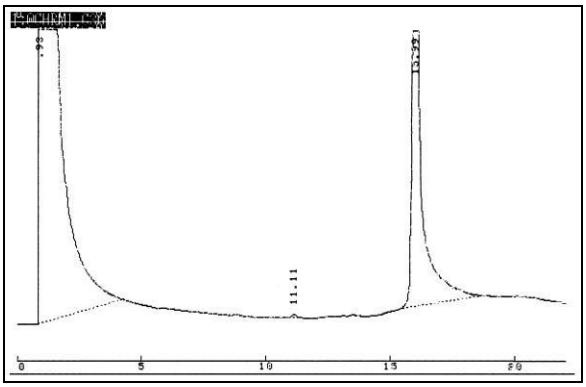


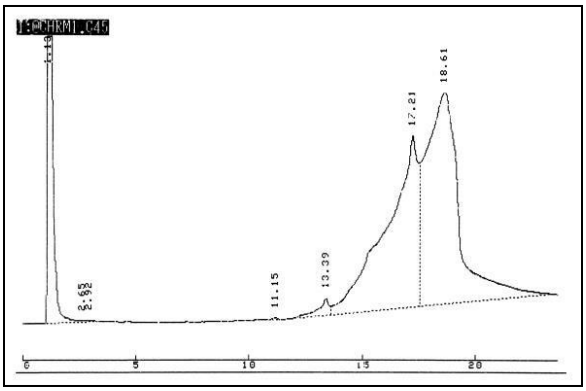
Octanoic



Lauric



Palmitic

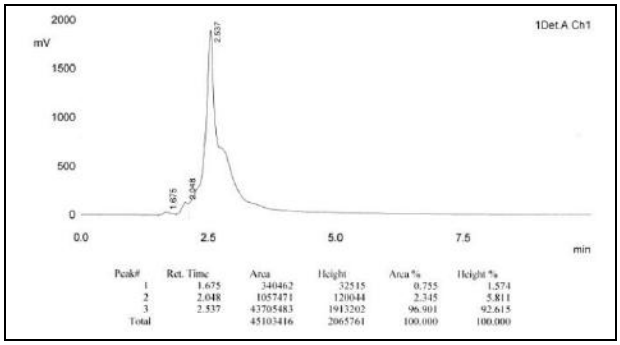


Oleic

*Fig.2: GLC chromatograms of standard fatty acid compounds.*

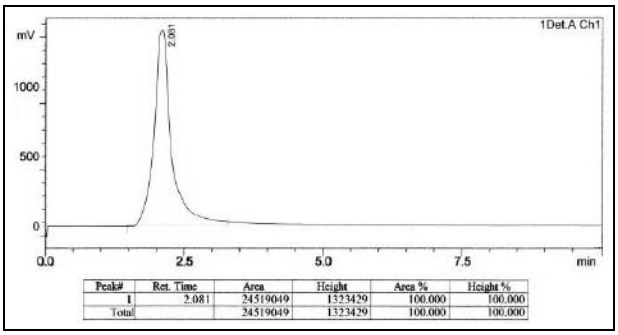
*Table.2: HPLC analysis of amla extracts of phenolic compounds.*

| Standard / Sample | Phenolic compound | | | |
|---|---|---|---|---|
| | Gallic acid | | Hydroquinone | |
| | 2.081 | | 2.530 | |
| | $R_t$(min) | Area % | $R_t$(min) | Area % |
| Fraction $A_2F_2$ | 2.048 | 2.345 | 2.537 | 96.901 |

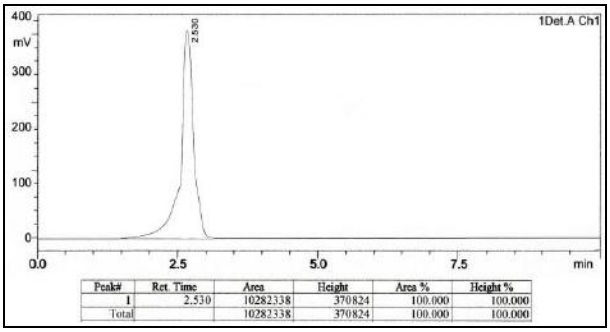


Fraction $A_2F_2$

*Fig.3: HPLC analysis of phenolic compounds present in amla extract.*



Gallic acid



Hydroquinone

*Fig.4: HPLC analysis of standard phenolic compounds.*

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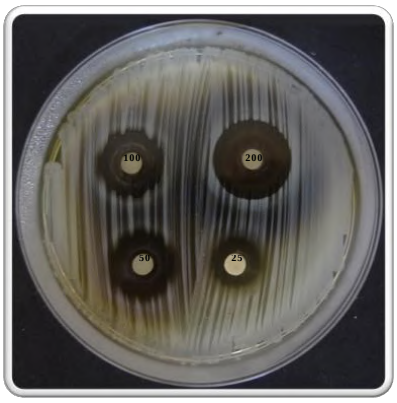
*Table.3: Antibacterial activity of fatty acids and phenolic compounds of amla in some pathogenic bacteria (mm).*

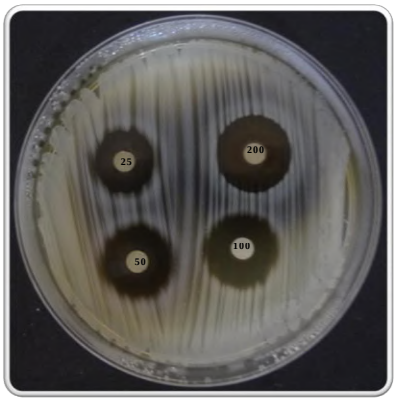
| Microbial species | Fatty acids extracts Phenolic compound extracts | Concentration of extracts ($mg/c^3$) | | | |
|---|---|---|---|---|---|
| | | 200 | 100 | 50 | 25 |
| *Staphylococcus aureus* | $A_1F_2$ | 25 | 20 | 19 | 15 |
| | $A_2F_2$ | 25 | 20 | 16 | 15 |
| *Salmonella typhimurium* | $A_1F_2$ | 24 | 20 | 16 | 10 |
| | $A_2F_2$ | 22 | 20 | 19 | 14 |
| *Escherichia coli* | $A_1F_2$ | 27 | 20 | 15 | 11 |
| | $A_2F_2$ | 26 | 15 | 11 | 9 |

*Table.4: Antibacterial activity inhibition zone (mm) using the standard antibiotics.*

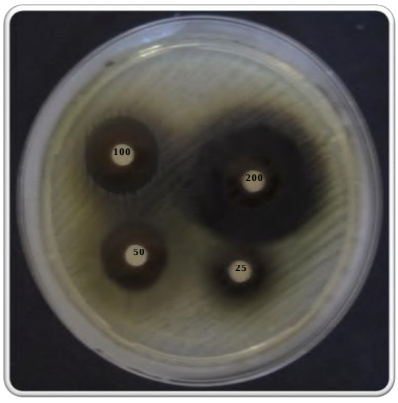
| Bacteria / Antibiotics | *Staphylococcus aureus* | *Salmonella typhimurium* | *Escherichia coli* |
|---|---|---|---|
| Gentamicin (CN) 10 meg | 21 | 19 | 25 |
| Amikacin (Ak) 10 meg | 15 | 15 | 16 |
| Ciprofloxacin (cip) 10 meg | 24 | 18 | 26 |




1. Effect of $A_1F_2$ on *Staph. aureus*




2. Effect of $A_2F_2$ on *Salmonella typhimurium*




3. Effect of $A_1F_2$ on *E. coli*

*Photos 1-3. Antibacterial activity of some fatty acids and phenolic compounds in amlaextracts on bacteria under study.*

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