CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF MANY FATTY ACIDS FROM FLOWERS OF *HIBISCUS SABDARIFFA* L. AND ITS INHIBITORY EFFECT ON SOME PATHOGENIC BACTERIA

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ABSTRACT

Gas-liquid chromatography (GLC) method was used for the qualitative and quantitative analysis of seven fatty acid compounds in the petroleum ether, chloroform and fractions (Chloroform-Ethanol) from (Aceton, Ethanol and also aqueous extract after using column chromatography of the crude extracts of *Hibiscus sabdariffa* flowers. Fatty acids were investigated as followings: Lauric, Palmitic, Linoleic, Stearic, Oleic, Heptanoic and Octanoic acids. The major component of fatty acids content was palmitic and Linoleic acids, respectively while the other fatty acids were identified in a trace amounts. All extracts under study were tested against some pathogenic bacterial of human, the grame positive were (*Corynebacterium diphtheria, staphylococcus aureus, staphylococcus capitis*), and the gram negative were (*Pseudomonas aurogenosa and protus merabeles*) from the all extracts, the fraction of (Chloroform-Ethanol) (H_3F_1) gave the highest effect as a result of containing of all the fatty acids except Heptanic acid, and it gave inhibition range: (26-34 mm) which was comparisons with authentic samples at concentration of Gentamicin (CN) (10 mcg/disc), Amikacin (AK) (10 mcg/disc), Tobramycin (TOB) (10 mcg/disc). As the effect of fatty acid extracts of Rosella flowers exceeded selected antibiotics under study. The combination of these fatty acids in H_3F_1 extract showed higher synergistic bactericidal effect against all bacteria under study.

Keywords: *GLC analysis, Fatty acid, Hibiscus sabdariffa extract, Antibacterial effect.*

1. INTRODUCTION

*Hibi*scus sabdariffa L. (Family Malvaceae) commonly known as Roselle, red sorrd, or karkade, is widely grown in Africa, South East Asia, and some tropical countries of America[1]. The freshy flowers provide a short drink consumed as a cold or hot beverage[2,3]. The daily consumption of this beverage, called "flor de Jama: ca" in Mexico[4]. And sobo in Nigeria[1] is high because of the sensation of freshness conveyed. The calyces are rich in acids and pectins, analysis of calyces has shown the presence of crude protein and minerals such iron, phosphorus, calcium, manganese, aluminium and also the fat[4]. Their water soluble polysaccharides have been isolated from the flower buds of *Hibiscus sabdariffa* neutral polysaccharides composed of arabinans and aribinogalactaus[5,6]. Roselle is an important source of vitamins, minerals, and bioactive compounds, such as organic acids. Phytosterole and polyphenol, some of them with antioxidants properties[7]. The main phenolic content in the plant consist of anthocyanins like delphinidin-3-glucoside and cyanidin-3-sambubioside, other flavonoids like gossypectin, hibiscetin and their respective glycosides; protocateauic acid, eugenol and sterols like β-sitoesterol and ergeoesterol[3]. Some researchers have focused on Rosella water extracts[8,9] while others have employed an organic soluent to extract possible bioactive compounds[10]. Indeed the different extraction techniques complicate comparisons among studies.

Moreover, different varieties of *Hibiscus sabdariffa* have been analyzed, and as far as we know, little has been published regarding the composition of Iraqi *Hibiscus sabdariffa*. The aim of this work was to find the quality and quantity of many fatty acids content in the flowers from *Hibiscus sabdariffa*, and also to study the effect of these fatty acid on some pathogenic bacteria. Aqueous-methanolic extract of *Hibiscus sabdariffa* calyces have been found to exhibit antibacterial activities against *Staphylococcns aureus*, *Bacillus stearothemophilus*, *Micrococcus luteus*, *Serratia mascences*, *Clostridium sporogenes*, *Escherichia coli*, *Klebsiella pneumonae*, *Bacillus cereus* and *Psedomona fluorescence*[11]. Nwaiwn[12] was showed that the antimicrobial properties of *Hibiscus esculentus* seeds, compare favourably with those of already popular *Moringa ofeifera* seeds. The present results support the use of this plant *Hibiscus sabdariffa* in the treatment of disease like abscesses, bilions condition, cancer and coughs in traditional medicine and also suggest the possibility of isolating antibacterial and anticancer agent for *Hibiscus sabdariffa*[11]. The plant extract showed potential antimicrobial activity against the *Staphylococcus aureus*, *Bacillus spp*. *K. pneumonia proteus spp*. *E. coli Ps aerughosa*, *Entecrococcus spp*.[13]. The previous study of Al-Mamum and co-workers[14] were showed designed to investigate the antibacterial, cytotoxic and insecticidal activities of the methanol (85%) extract of fruits of *Hibiscus sabdariffa*. The study of El-Kamali and co-workers[15] showed *E. coli* with a higher resistance of the *Hibiscus sabdariffa* extracts where as *Pseudomonas aeroginosa* is more sensitive[16].

1. MATERIAL AND METHODS

Roselle flowers: Sample preparation, *Hibiscus sabdariffa* L., flowers packed bags was acquired from a local supermarket in Mosul-Iraq and also classified by Mr. Talal Taha. (a director of the medicinal plant project in the Mosul Dam Project).

The material was cleaned, dried, and also grinded.

Preparation of plant extracts: A batch of 50 gm of the grinded flowers of Roselle was soxhlet extracted for 6-8 hrs. with 1 L of four solvents (petroleum-ether: 60-80°C chloroform, acetone, and also ethanol), water was also used at room temperature as aqueous solvent.

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 acetone, ethanol and aqueous crude extract: 300 mg of each crude extracts 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 (petroleum ether: chloroform) with concentration of 10% V/V intervals, followed with continuous elution with (chloroform: ethanol)[17].

Saponification of pet-ether extract was refered to (H_1) and also (H_2) for chloroform extract. From acetone crude extract was fraction ated to chloroform-ethanol which saponified to get (H_3F_1), while the ethanol fraction after saponification was also refered to (H_3F_2).

From the crude ethanolic extract was fractionated to (chloroform-ethanol) which also saponified to get (H_4F_3).

Moreover, the aqueous crude extract was fractionated to (chloroform-ethanol) and after saponification was carried out to get (H_5F_1).

The fractions were kept in dark place for further studies.

Alkaline hydrolysis: Petroleum-ether, chloroform extracts and fractions of (chloroforms: ethanol) as a resulted from column chromatography A mixture of 10 gm of each pet-ether, chloroform extracts and also the fractions; (chloroforms: ethanol) from acetone, ethanol, and aqueous extracts and 100 ml of 7.5 M of solution of KOH in methanol: water (3:2) was refluxed in a round bottom flask for 90 min. at 100°C. The suspension was allowed to cool at room temperature and 100 ml of distilled water added. The crude was extracted with diethyl ether (2×100 ml) to remove unhydrolysed lipid. The hydrolyse was acidified with 20% (V/V) conc. H_2So_4 up to PH=2. The liberated fatty acids were extracted with diethyl ether (2×100 ml).

The combined extracts were washed with water, dried over anhydrous sodium sulfate, filtered and concentrated in vacuo to give 1.2 gm of crude fatty acids[18].

Preparation of Methyl Esters: 0.1 ml of acetyl chloride was added to 25 ml dry methanol with stirring. A sample of 0.5 ml of dry fatty acids was added to the above mixture then boiled under reflux in water bath for 20 min. dry closed system, cooled then used for analysis by GLC[19].

GLC-Analysis: The fatty acid methyl esters can be obtained either directly by transmethylation of the parent lipids by refluxing then for 90 min. with methanol –benzene. H2So4 (20:10:1) or from the free acids by the acetyl chloride – methanol procedures.

The esters were analysed by GLC on a Packard 419 equipped with a danl flame ionization detectors held of 270°C. A(2.12m×2m) international dimeter column packed with 3% Silar 10°C on Gas chrom Q (100-120) mesh was held at 160 initially, then raised at 5°C/min to 200°C.

The identification of fatty acids was determined by references to a standard of a known composition[20].

The studied bacteria was selected of five types of pathogenic bacteria that infect different parts of the human body is positive for Gram stain, *Staphylococcus aureus*, *Corynebacterium diphtheria Staphylococcus capitis* and negative for Gram stain is *Pseudomonas aurogenosa* and *Proteus merabeles*.

All the pathogenic bacteria under study were identified in to Biology Dept., College of Science, University of Mosul.

Antibacterial activities of prepared extracts of *Hibiscus sabdariffa*: The antibacterial effect of these extracts were tested on all studied bacteria according to the method of modified Kirby-Bauer[21] in which the inoculums were prepared in nutrient broth and incubator at 37°C for (18-24) hrs.

The density of the tested suspensions was adjusted to that of the 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 surface of the application. Finally, the inoculums were left to dry for a few minutes later 50 ml of each of the prepared extracts call the extracts were dried and re-dissolved in DMSO were placed in walls 6 mm in diameter which were done on the inoculated plates, the antibiotics 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 that of the standard antibiotics (Gentamicin (CN) 10 mcg, Amikacin (AK) 10 mcg, Tobramycin (TOB) 10 mcg). Also, we are used various concentration (800-12.5) mg/cm^3 of plant extracts under study.

The extract that produced zones of inhibition was further studied using different concentration of this extract ranged from (800-12.5) mg/ccm^3[22].

2. RESULTS AND DISCUSSION

During the phytochemical screening of the *Hibiscus sabdariffa* composition, the seeds were also contained fatty acids as follows, Myristic (0.5%), Palmitic (16.2%), Stearic (4.1%), Oleic (9.4%), Linoleic (67%), linolenic (2.2%) acids[4].

Many authors were refered to find the fat as a one of constituents of the flowers of *Hibiscus sabdariffa* that make our to do many steps to investigate fatty acids content[4].

The table (1) and figure (1 and 2) were showed the presence of some fatty acids in the flower of *Hibiscus sabdariffa* in different extracts, so the H_1 (Saponified of pet-ether extract and methylation) extract was contained palmitic (0.27%) and Linoleic (0.7%) acids.

Where H_2 (Saponified of chloroform extract and methylation) was contained palmitic (0.04%), Linoleic (0.004%), Stearic (0.008%), also Heptanoic (0.003%) and Octanoic (0.011%) acids.

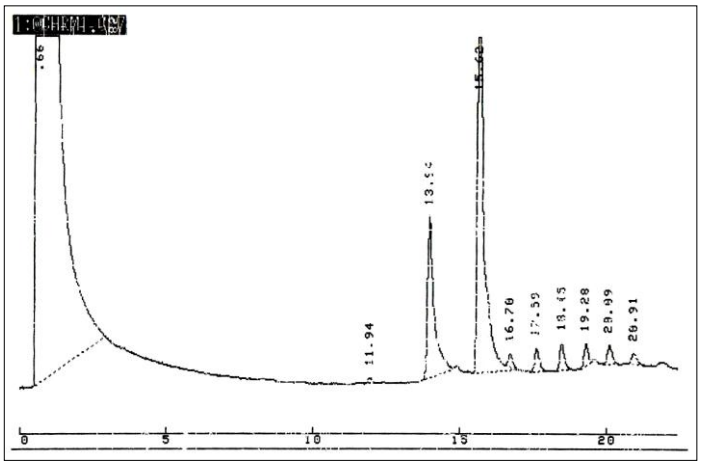
The other fractions which were carried out from the column and saponification took place to release fatty acids and methylation was accured to get GLC-analysis which indicated as followings:

The fraction (H_3F_1) is contained Lauric (0.005%), Palmitic (0.002%), Linoleic (0.002%), Stearic (0.002%), Oleic (0.0063%) and Octanoic (0.004%) acids. While the fraction (H_3F_2) was contained Linoleic (0.192%) and Oleic (0.013) acids. The fraction (H_4F_3) was also contained Lauric (0.018%), Palmitic (0.01%), Linoleic (0.007%), Oleic (0.013%) and Octanoic (0.068%) acids. Finally, the fraction (H_5F_1) was contained Lauric (0.003%), Palmitic (0.001%) and also Octanoic.

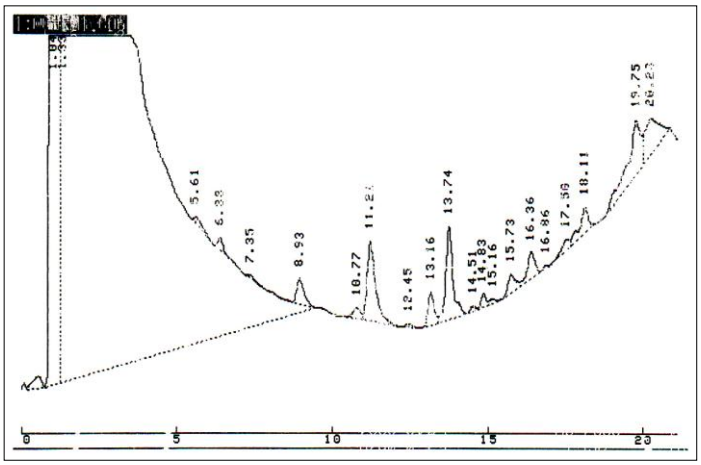
From this table which indicated that fraction of H1 which was contained a high concentration of palmitic (0.27%) and Linoleic acids (0.704%) and this was confirmed that this fraction extracted more lipid content Linoleic acid which also named as omega-6 and played an important role in the body and it combined with omega-3 gave many health benifite[23].

Table (1): GLC analysis of *Hibiscus sabdariffa* extracts of fatty acid compounds.

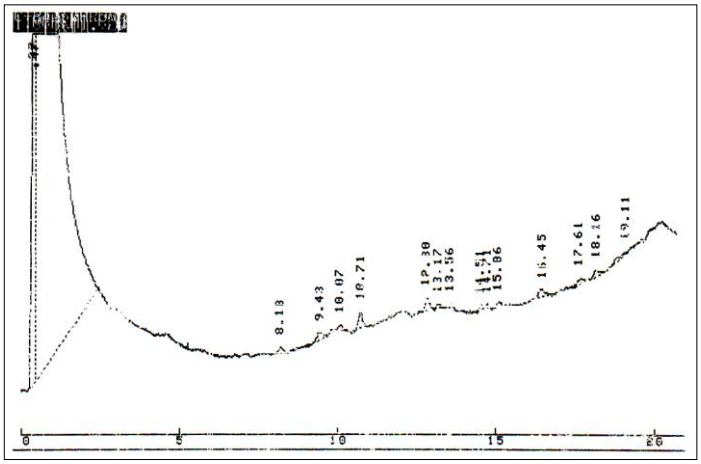
Extracts of *Hibiscus sabdariffa*	Fatty acid compounds													
	Lauric acid		Palmitic acid		Linoleic acid		Stearic acid		Oleic acid		Heptanoic acid		Octanoic acid	
	R_t(min)	Conc.	R_t(min)	Conc.	R_t(min)	Conc.	R_t(min)	Conc.	R_t(min)	Conc.	R_t(min)	Conc.	R_t(min)	Conc.
Extract (H_1)	-	-	13.948	0.2766	14.82	0.7046	-	-	-	-	-	-	-	-
Extract (H_2)	-	-	13.741	0.0415	14.517	0.0045	15.738	0.0085	-	-	6.38	0.0036	8.938	0.0111
Fraction (H_3 F_1)	9.439	0.0057	13.562	0.0028	14.515	0.0022	15.062	0.0024	16.4	0.0063	-	-	8.186	0.0041
Fraction (H_3 F_2)	-	-	-	-	14.048	0.1927	-	-	15.712	0.0131	-	-	-	-
Fraction (H_4 F_3)	9.467	0.0186	13.603	0.01	14.537	0.007	15.425	0.0131	-	-	-	-	8.201	0.0680
Fraction (H_5 F_1)	9.458	0.0033	13.221	0.0016	-	-	-	-	-	-	-	-	8.902	0.003
Standards	9.47		13.63		14.57		15.45		15.94		6.925		8.309	



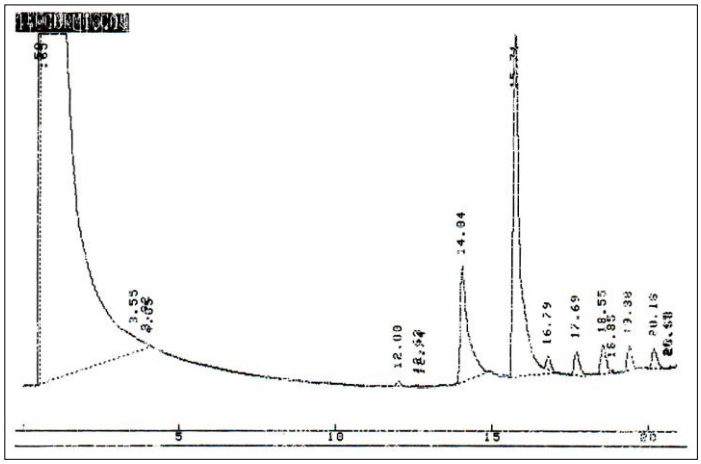
(H1) Extract



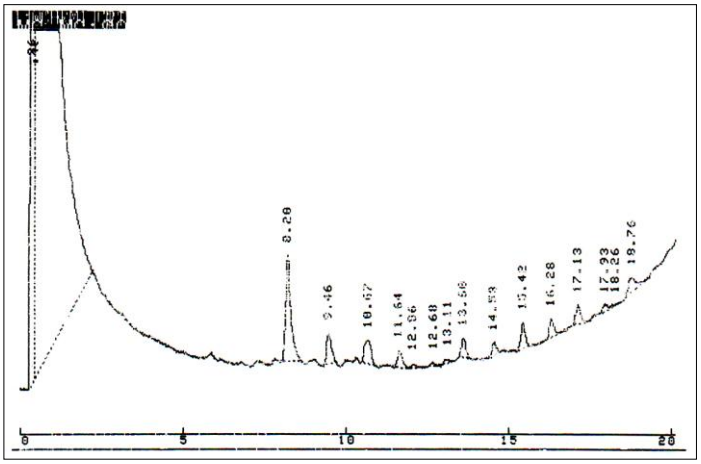
(H2) Extract



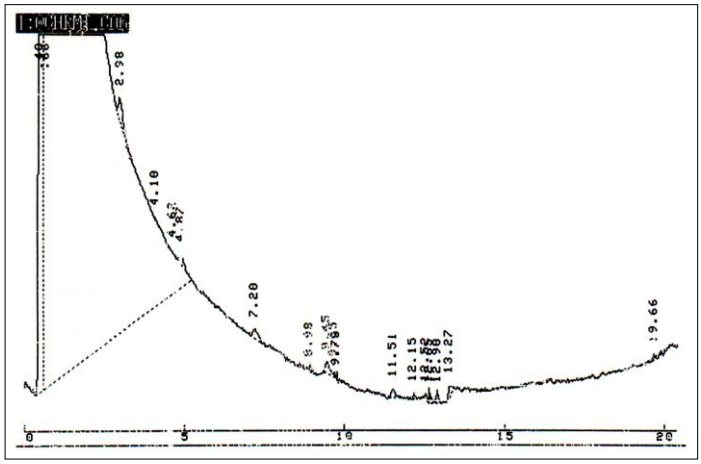
Fraction (H3F1)



Fraction (H3F2)

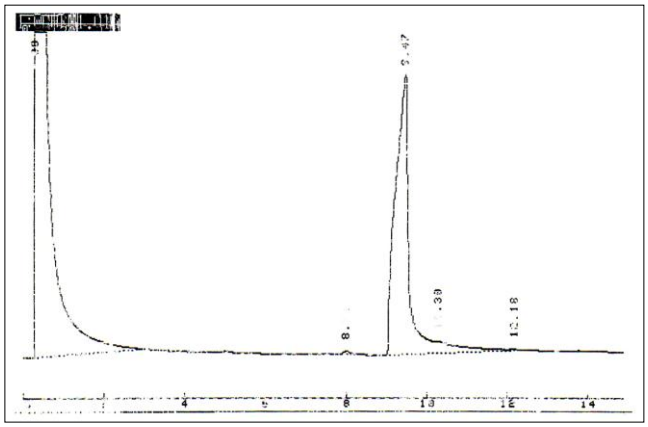


Fraction (H4F3)

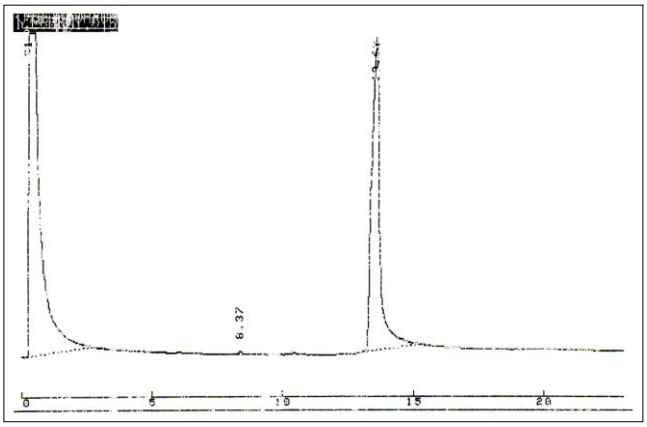


Fraction (H5F1)

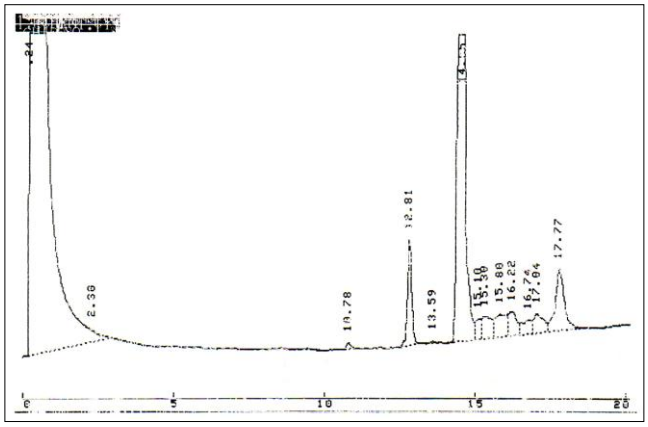
Figure (1): GLC Chromatograms of fatty acid compounds presented in *Hibiscus sabdariffa* extracts.



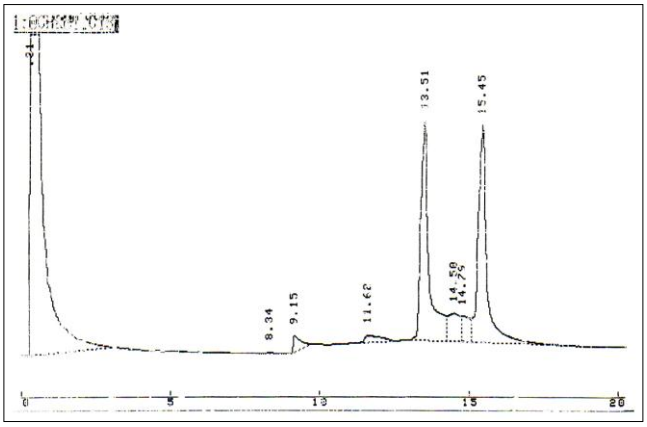
Lauric acid



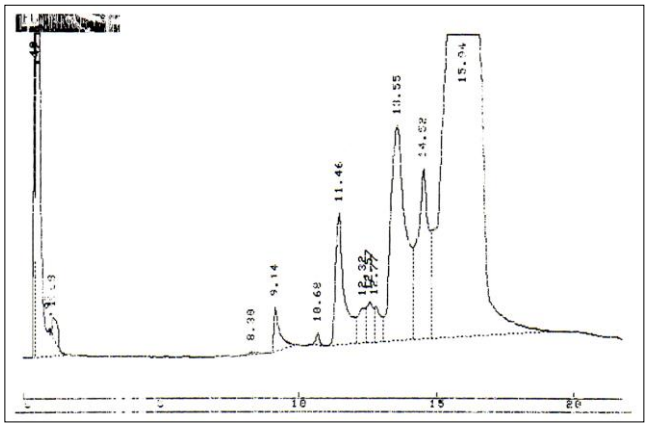
Palmitic acid



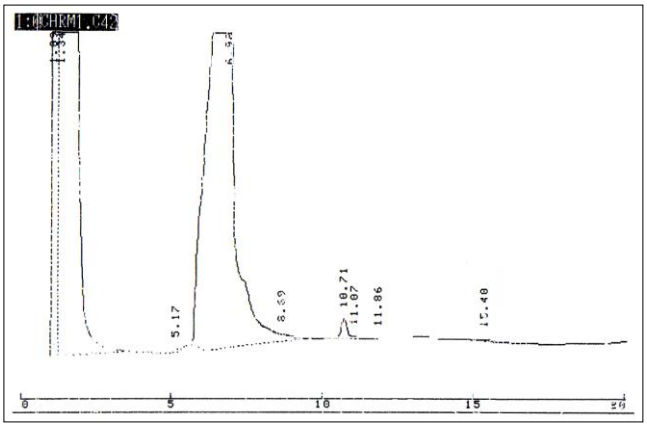
Linoleic acid



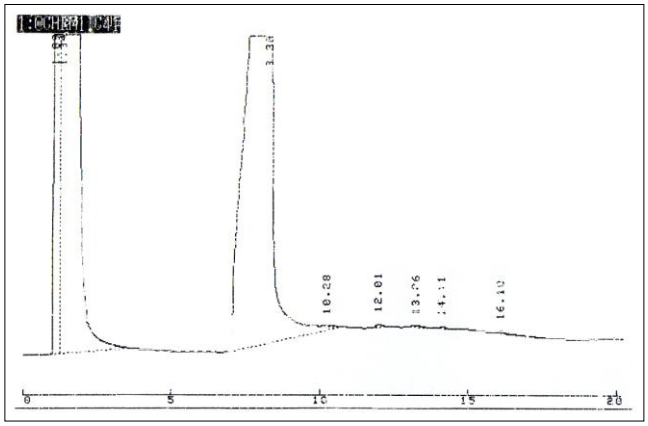
Stearic acid



Oleic acid



Heptanoic acid



Octanoic acid

Figure (2): GLC Chromatograms of standard fatty acid compounds.

The table (2 and 3) and image (1-10) showed the antibacterial activity of *Hibiscus sabdariffa* extracts against some pathogens bacteria (*Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *Staphylococcus capitis*, *Coryanbacterum diphtheria*). All the fractions of the crude extracts of *Hibiscus sabdariffa* which almost containing fatty acid compounds gave antibacterial activity as compared with antibiotic compounds (Gentamicin (CN) 10 meg, Amikacin (AK) 10 meg, Tobramycin (TOB) 10 meg). Among the eight fatty acids which were presented in the (Chloroform-ethanol) fraction of a crude acetone extract (H_3F_1), was only found to exhibit strongest antibacterial activites against all the bacterial used under study. From previous studies, Olaleye[11] was used *Hibiscus sabdariffa* extracts and his result was showed the antibacterial effect on some groups of pathogenic bacteria within *Staphylococcus aureus* and *Pseudomonas aeurogenosa* that were under study, and this was consistent with our study. Our interesting to note that the plant extracts were able to inhibit the growth bacteria under study more than antibiotic that be used. The fraction (Chlorofrom-ethanol) in a crude acetone extract (H_3F_1) was containing of six fatty acids compounds (Lauric, Palmitic, Linoleic, Stearic, Oleic and Octanoic acids). From all investigated four fatty acids compounds, Palmitic, Linoleic, Stearic and Octanoic acids were showed to exhibit the strongest antibacterial activity. Fatty acids showed stronger antibacterial activity against *Pseudomonas aerogenosa* by increasing the permeability of the inner bacterial membrane and a dissipation of the membrane potential[24,25]. So, these results showed the combination and which were presented with higher concentration of these six fatty acids compounds demonstrated synergism for their antimicrobial activity against all the bacterial under investigation[25,26]. Finally, also two previous studies[27,28] were carried out of using the seeds of *Hibiscus sabdariffa* extracts that yielded of unsaturated fatty acids, Linoleic and Oleic acids, and can be used as a source of unsaturated fatty acids, so the saturated fatty acids were palmitic and stearic acids and this result was constent with our study especially related to above four mentioned fatty acid compounds.

Table (2): Antibacterial activity of many extracts and fatty acid separation of *Hibiscus sabdariffa* in some pathoginc bacterial (mm)

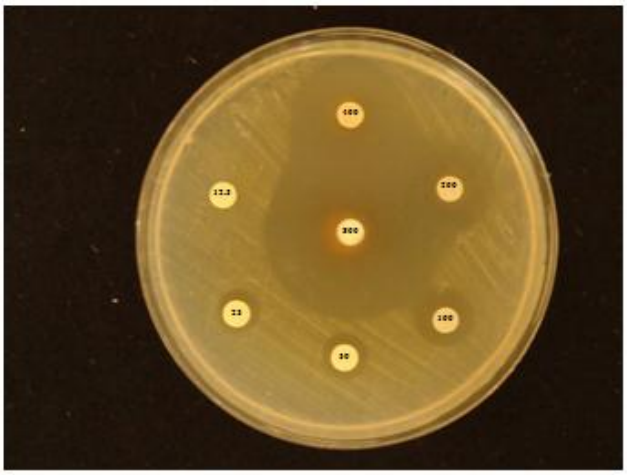
Microbial species	Type of fatty acids extracts	Concentration of extracts (mg/c^3)						
		800	400	200	100	50	25	12.5
Corynebacterium diphtheria	Fatty acid H_1	35	32	23	14	12	-	-
	Fatty acid H_2	20	16	14	14	12	12	7
	Fatty acid H_3 F_1 Acetone extract	32	26	22	22	10	9	9
	Fatty acid H_3 F_2 Acetone extract	26	21	20	14	12	10	8
	Fatty acid H_4 F_3 Ethanol extract	17	12	12	9	8	-	-
	Fatty acid H_5 F_1 Aqueous extract	15	16	16	-	-	-	-
Staphylococcus aureus	Fatty acid H_1	20	15	14	13	-	-	-
	Fatty acid H_2	15	13	11	8	18	15	8
	Fatty acid H_3 F_1 Acetone extract	34	24	24	16	18	13	13
	Fatty acid H_3 F_2 Acetone extract	30	25	21	19	17	11	7
	Fatty acid H_4 F_3 Ethanol extract	21	18	16	9	8	7	7
	Fatty acid H_5 F_1 Aqueous extract	17	12	12	8	-	-	-

Staphylococcus capitis	Fatty acid H_1	22	15	13	-	-	-	-
	Fatty acid H_2	16	10	11	8	18	14	8
	Fatty acid H_3 F_1 Acetone extract	32	24	22	16	13	16	16
	Fatty acid H_3 F_2 Acetone extract	31	25	22	19	16	12	8
	Fatty acid H_4 F_3 Ethanol extract	19	15	14	9	8	7	-
	Fatty acid H_5 F_1 Aqueous extract	16	15	10	9	7	7	7
Psendomonas aurogenosa	Fatty acid H_1	9	-	-	-	-	-	-
	Fatty acid H_2	10	9	10	9	8	-	-
	Fatty acid H_3 F_1 Acetone extract	27	21	19	13	14	13	12
	Fatty acid H_3 F_2 Acetone extract	32	24	20	18	16	12	-
	Fatty acid H_4 F_3 Ethanol extract	17	10	10	9	7	7	-
	Fatty acid H_5 F_1 Aqueous extract	16	10	7	8	7	-	-
Protus merabeles	Fatty acid H_1	20	-	-	-	-	-	-
	Fatty acid H_2	21	20	17	12	17	10	-
	Fatty acid H_3 F_1 Acetone extract	26	23	18	12	15	12	14
	Fatty acid H_3 F_2 Acetone extract	31	23	22	21	20	13	7
	Fatty acid H_4 F_3 Ethanol extract	15	16	14	9	-	-	-
	Fatty acid.H_5 F_1 Aqueous extract	13	13	12	9	8	8	-

(-) Resistance.

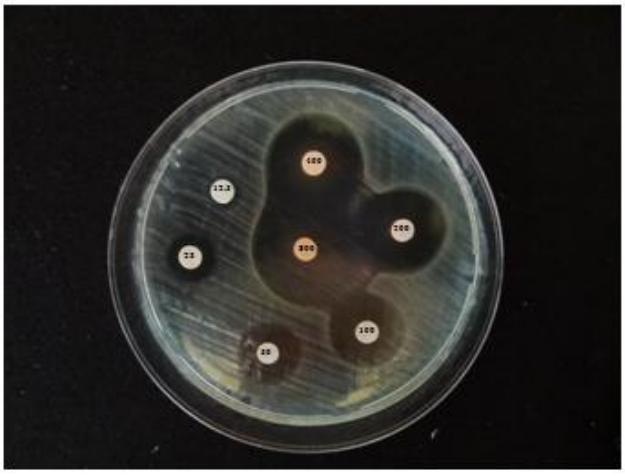
Table (3): Antibacterial activity inhibition zone (mm) using the standard antibiotics

Bacteria / Antibiotics	Gram positive			Gram negative	
	Corynebacterium diphtheria	*Staphylococus aureus*	*Staphylococcus capitis*	*Pseudomonas aurogenosa*	*Proteus merabeles*
Gentamicin (CN) 10 mcg	21	7	20	15	6
Amikacir (AK) 10 mcg	15	16	24	16	14
Tobramycin (TOB) 10 mcg	22	8	30	20	11

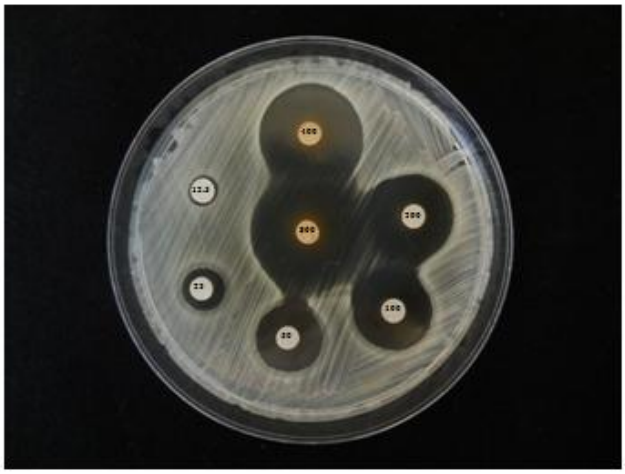


(1) Effect of H1 on *Coryanbacterium deftheria*

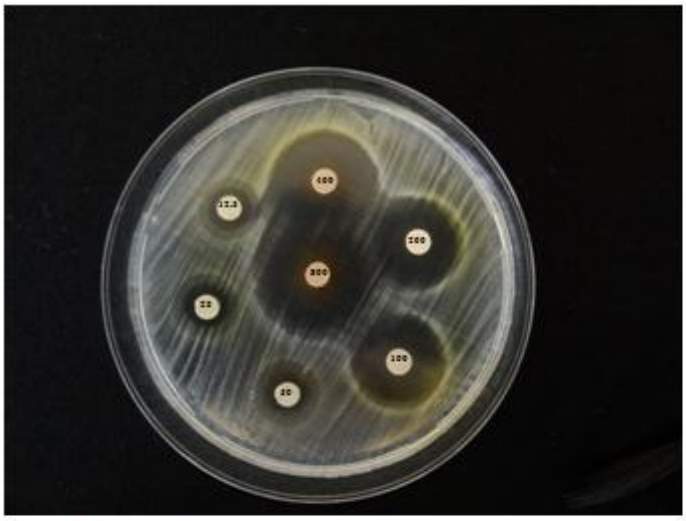
(2) Effect of H2 on *Staphylococcus capitis*



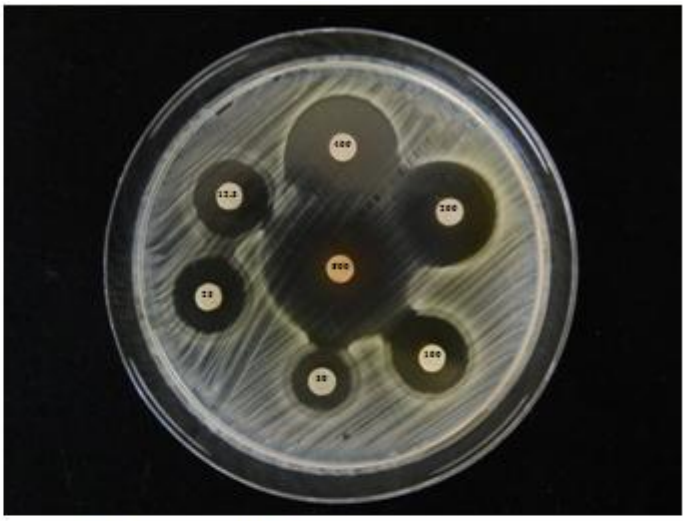
(3) Effect of H3F1 on *Pseudomonas aeruginosa*



(4) Effect of H3F1 on *Staphylococcus aureus*



(5) Effect of H3F2 on *Coryanbacterium deftheria*

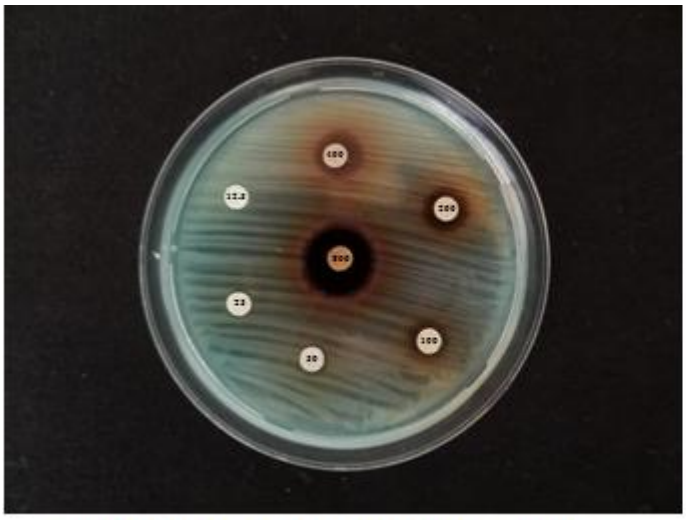


(6) Effect of H3F2 on *Staphylococcus capitis*

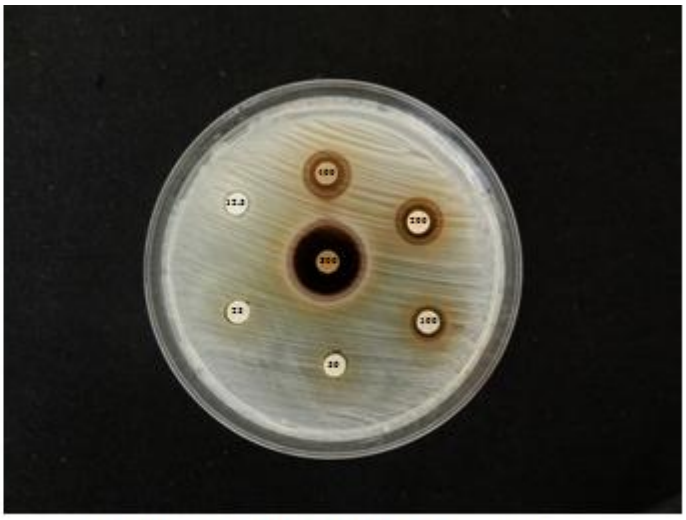
(7) Effect of H4F3 on *Pseudomonas aeruginosa*

(8) Effect of H4F3 on *Coryanbacterium deftheria*

Image (1-10): Antibacterial activity of some fatty acids in *Hibiscus sabdariffa* extracts on bacteria under study.



(9) Effect of H5F1 on *Pseudomonas aeruginosa*



(10) Effect of H5F1 on *Staphylococcus aureus*

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