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Original Research Article

Evaluation of anti-bacterial activity of *Polygonum plebeium* and *Euphorbia hirta* against *Pseudomonas aeruginosa*

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Abstract

This study investigated the extracts of *Euphorbia hirta* and *Polygonum plebeium* against human bacterial pathogen *Pseudomonas. aeruginosa* PAO1, B24 (isolated from wound infection of the arm, Cleopatra Hospital) with accession no.0748281. Among all tested solvents, ethyl acetate extract of *Euphorbia hirta* and *P.plebeium* was the most effective. Also the ethyl acetate extract of both medicinal plants has synergitic effect which increased the antimicrobial activity against *P. aeruginosa* PAO1, B24. MIC of plant extract *P.plebeium and E.hirta* against the growth of *P. aeruginosa* PAO1, B24 was found to be 12.5mg/m. Parameters controlling the antimicrobial activity of ethyl acetate extract of *E.hirta* and *P.plebeium* had been studied. It was more effective when incubated at 45°C for microbial strains *P. aeruginosa* PAO1, B24; pH5 was more effective against *P. aeruginosa* PAO1, B24; Dark condition for 24hr was effective against *P. aeruginosa* PAO1, B24; Static condition had slight effectiveness; Xylose exhibited the highest antimicrobial activity against *P. aeruginosa* PAO1, B24; it was not affected by the different types of amino acids and no metallic ions was effective against *P. aeruginosa* PAO1, B24. Also no vitamins were effective on the microbial strain under study.

Keywords: *Euphorbia hirta*, Medicinal plants, *P.aeruginosa*, *Polygonum plebeium*

INTRODUCTION

Despite the huge number of anti-microbial agents for various purposes that already exist, the search for new drugs is a cutaneous task since the target micro-organisms often develop new genetic variants which subsequently become resistant to available anti-microbial agents and effective lifespan of any anti-biotic is these limited. The world's attention is now increasingly directed towards a plant sources for developing anti-microbial drugs. Natural products are considered safer than synthetic ones (Nascimento et al., 2000) Plants are used medicinally in different countries and are a source of many potent and powerful drugs. A wide range of medicinal plant parts is used to extract raw drugs and they have varied medicinal properties (Okwuosa et al., 2012).

Excessive use of antibiotics led to bacterial adaptation, resulting in the development of multi-drug resistance in bacteria. This has limited the efficacy of antibiotics, warranting alternative strategies to eliminate the microbial infections. Since the synthetic drugs are expensive and lead to awful side effects, the use of medicinal plants is getting importance due to better results and less or no side effects. The active biological compounds are prepared from different parts of the plants (Mathivanan et al., 2014).

E. hira belongs to the family Euphorbiaceae, it is considered as a herb found in tropical countries. Its flowers are small, numerous and crowded together in dense cymes (dense clusters in upper axils).Moreover, the plant is also used to treat infections of the skin and

mucous membranes, including warts, scabies and tinea Sandeep et al., 2009. Tawa-tawa, gatas-gatas is abundant in open grasslands and waste places. The scientific name is *E. hirta*, it is classified as weed and is a native in India (Lorna et al., 2015). *E. hirta* plays an important role in the management of diabetes mellitus, especially in developing countries. (Mathivanan et al., 2014)

Heshouwu, derived from the root of *P. multiflorum* (Fallopia multiflora), is widely used in the Chinese medicine market as a traditional tonic. The emergence of Heshouwu material with a human shape reflects a pursuit of its supplementing effects. However, reports of Heshouwu toxicity have repeatedly surfaced in recent years, attracting widespread concern by the Chinese Pharmaceutical Association (Hong-Yu et al., 2016)

In traditional Chinese medicine Polygonum *cuspidatum* is widely used, the larger production, and in the clinical application. By reviewing the relevant literatures in recent years, the chemical constituents and pharmacological effects of *Polygonum cuspidatum* were sorted and summarized, not only study the medicinal mechanism of *P. cuspidatum*, but also provide the theoretical basis for the medicinal development, clinical treatment and comprehensive utilization of *P. cuspidatum* (Guo et al., 2018).

P. plebeium is a winter annual weed, from family Polygonaceae, Polygonum is from the Geek, poly meaning many and gony meaning knee and refers to many nodes on the stems. Prostrate, mat-forming herb. Stems reddish-brown, up to1m long, becoming somewhat woody at the base; leaves subsessile, leathery and hairless its color ranges from dark green to reddish-brown and the margin rolled under, it occurs in disturbed habitats that frequently are flooded, such as banks, ditches, and rice fields. It is used as a vegetable in food in some locations. (Schardl et al., 2007)

The dissemination of pathogenic bacteria in the community and healthcare setting is of great concern and associated with high mortality and morbidity (Bakthavatchalam et al., 2017). Reports emanating in Middle East countries revealed increasing rates of incidence of pathogenic bacteria in Egypt (Abdel-Maksoud et al., 2016). *P. aeruginosa* is a gram-negative, rod-shaped bacterium. *P. aeruginosa* is a "multi-drug resistant (MDR) pathogen" recognized for its association with serious variable diseases in plants and animals (Balcht et al., 1994). *Pseudomonas aeruginosa* can, in rare circumstances, cause community-acquired pneumonias as well as ventilator-associated pneumonias (Chua et al., 2014).

Since the synthetic drugs are more costly and lead to side effects, the usage of herbal medicines is getting importance due to promising results and less or no side effects. Active biological compounds which have great potential as antimicrobial agents are prepared from various parts of medicinal plants.

MATERIALS AND METHODS

Plant materials

The leaves of nine types of medicinal plants were collected from different locations as follows: *E. hirta* L (EH) and *Forsskaolea tenacissima* (FT) were collected from the Garden of Faculty of Agriculture, Al-Azhar University. *P. plebeium* (PP), *Lolium temulentum* (LT), *Minuartia cumberlandensis* (MC), *Silene rubella* (SR), *Gemellina hystrix* (GH) were collected from the Ekhnawy Tanta region, Egypt and were kindly authenticated by Dr. Abdo Marey, Asst. Prof. of Botany, Department of Botany and Microbiology, Faculty of Science, Al-Azhar University. While *Thymus decussatus* (TD) was collected from Sant Catren and *Kalanchoe marmorata* (KM) collected from Orman garden, Egypt. Voucher specimens are deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. These species were air-dried, powdered and kept in tightly closed amber colored glass containers and protected from light at low temperature as possible (freezing).

Test organisms used

The most resistant bacteria isolat*e P.aeruginosa*, B24 was selected from 58 bacterial isolates. *P.aeruginosa*, B24 isolated from wound infection of the arm, Cleopatra Hospital, Cairo, Egypt. The bacterium isolate was cultivated in nutrient agar medium (NAM) (Hi Media, India).

Identification of the selected bacterial isolate by 16s rRNA

The most resistant pathogenic isolates were identified based on16s ribosomal RNA gene sequence. The sequences obtained will aligned by the computer program (Fraser et al., 2002). Identification was carried out by Sigma Scientific Services Company, Cairo, Egypt.

Preparation and Extraction of plant material

Ethanolic extraction of plant material was carried out according to the method described by (Janakat et al., 2005) with some modifications.

About 100 gm powder of *E. hirta* and *P. plebeium* were completely extracted with methanol in a Soxhlet apparatus. The extracts were concentrated under reduced pressure in a rotary evaporator, until it is dried.

It was suspended in water, filtered, purified and partitioned into different fractions according to a general procedure elaborated for phenolics (Janakat et al., 2005).

Antimicrobial activity of selected medicinal plants against selected bacterial strains

The antimicrobial assay was performed using the agar well diffusion method described by (Betina, 1957) for ethanolic extract of 9 medicinal plants. Only 10 µl of each target resistant strains were inoculated into 100 ml warm, nutrient agar medium (45°C).The media were poured into sterile plates and left for solidification. Each plate is called a seeded plate. The seeded plates with target organisms were cut by sterile cork porer to make holes (11 mm in diameter). Only 100 µl of the plant extracts was transferred into each hole under aseptic conditions. Then, the plates were kept in refrigerator for 2 hours before incubation to permit diffusion of the metabolites before the growth of the resistant pathogenic bacterial strains takes place.

Subsequently, the plates were incubated at 37°C for 24 hours and then examined for antimicrobial activities. Duplicate plates were used for each test micro-organism Triplicates of measurements were carried out and the mean of inhibition zone diameter was calculated.

Determination of the MIC of *E. hirta* and *P. plebeium* extracts against selected strain

The minimum inhibitory concentration (MIC) had been determined by agar diffusion method according to (Kavanagh, 1972).

Different concentrations of plant extracts were prepared by using distilled water. The different concentrations used were 100, 50, 25, 12.5 and 6.2 (mg/ml). Then, 100 µl of different concentrations was placed in the holes. The experiment was carried out in duplicate. The plates were incubated at 37°C for 24 hours. The response was observed as a clear zone (mm) around the holes.

Parameters controlling the growth of the selected strain in relation to mixed ethyl acetate extract of *E. hirta* and *P. plebeium*

The following parameters were carried out to optimize the antimicrobial activity against the resistant pathogenic bacterial strains. Agar diffusion method was used in this respect. After determining each parameter, the best result was applied in the subsequent parameters. Triplicates were used for each particular parameter. The antimicrobial activity was assayed.

Different incubation temperatures

In this respect, mixed of ethyl acetate extracts of *E. hirta* and *P. plebeium* was incubated at different temperatures (10.0, 25.0, 35.0, 45.0, 100 and 121° C) for several incubation periods (0, 10min., 1, 6, 24, 48 hr.) and then used against the resistant pathogenic strain.

Different pH's values

The pH of plant extracts was measured (pH 5.8) and registered. Then the mixture of plant extracts was modified to different pH's(2.0, 3.0, 5.0, 8.0 and 9.0) and incubated at 37°C for several incubation periods (Zero, 10min., 6, 24 and 48 hr.) and then used against the selected resistant pathogenic strain, in order to determine the best pH and incubation time .

Dark and light

In this experiment, the mixture of ethyl acetate extract of *E.hirta* and *P. plebeium* was subjected to light and dark for 24 hours. And then was used against the resistant pathogenic strain, in order to examine the effect of dark and light on the antimicrobial activity of plant extracts.

Static and shaken conditions

The mixture of ethyl acetate extract of *E.hirta* and *P. plebeium* was used under static and shaken conditions (50 and 150 rpm) condition against the resistant pathogenic strain.

Different carbon sources

Different carbon sources were added at 1% to the ethyl acetate extract of *E. hirta* and *P. plebeium* and then the plant extract was used against the resistant pathogenic strain in order to determine the best carbon source in combination with the plant extract can affect the resistant pathogenic strain effectively. The carbon sources used were xylose, glucose, fructose, lactose, maltose, carboxy methyl cellulose (CMC), galactose, sucrose and starch.

Different amino acids

Different amino acids (DL-Tryptophan, L-Lysine, L-Arginine, DL- Asparagine , L-Serine, L-Cysteine, L-Alanine, L-Histidine, L-Leucine, L-Phenyl alanine, DL-Threonine and Glycine). Then the previous amino acids were added separately at equimolecular nitrogen located in sodium nitrate to mixed ethyl acetate extracts of

E. hirta and *P. plebeium* and then used against the resistant pathogenic strain, in order to determine the best amino acid which can increase the efficiency of the antimicrobial activity of the plant extracts.

Different metallic ions

Different metallic ions were used viz. (ZnSO4, CaCl2, MgSO4, CoCl2, KCl and EDTA) in three different concentrations 50,100 and 200 (ppm) .They were added to mixed ethyl acetate extracts of *E.hirta* and *P. plebeium* and were used to detect the best metallic ion by which the extracts can affect the resistant pathogenic microorganisms.

Different vitamins

In this respect, different types of vitamins were used, including nicotinic acid, riboflavin, ascorbic acid and inositol in three different concentrations (50, 100 and 200 ppm) were added to the mixed ethyl acetate extracts of *E. hirta* and *P. plebeium* and then the extracts was used against the resistant pathogenic strain.

Separation and purification of the antimicrobial substance from *E. hirta, P. plebeium*

Column chromatography

A glass column of (2.5 x 50) cm diameter was used for such purpose as shown in plate (22). The column was packed with activated silica gel as follows: The silica gel was mixed with chloroform as an eluting solvent. A glass rod was often used to stir the slurry to prevent air bubbles from being trapped with the slurry. Once the slurry gets homogenous, it was poured cautiously into the empty column and the column was left overnight until the silica gel was completely settled. The silica gel was occupied 30 cm of the column.

The elution gradient was added as follows:
1) Chloroform: ethyl acetate (95: 5)
2) Chloroform: ethyl acetate (90: 10)
3) Chloroform: ethyl acetate (85: 15)
4) Chloroform: ethyl acetate (80: 20)
5) Chloroform: ethyl acetate (75:25)

85, 80 fractions were collected from *E. hirta* and *P. plebeium* respectively each of 20ml. All fractions were tested for their activity using agar diffusion method against resistant pathogenic strains. The separated fractions were examined by TLC assay for their purity.

Characterization of the most active pure compounds

The following spectroscopic techniques have been used in this experimental work to predict the empirical chemical structure and the suggested molecular formula as well as nomenclature of the most active compound.

Mass (MS) Spectrum

Mass spectrum was carried out on Direct part DI-50 to the mass analyzer in Shimadzu GC/MS-QP5050 (Germany) at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The mass spectroscopy system was used to confirm the purity of the compounds.

Infra red (IR) spectrum

The Infra-Red absorption spectrum was obtained in the solid state in the form of KBr discs and recorded using FTIR Shimadzu spectrophotometer (4000-400cm) (Germany), Chemistry Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt.

RESULTS AND DISCUSSION

The main objective of this study is to investigate the antibacterial activity of different extracts of *Euphorbiahira* (Euphorbiacea) and *P. plebeium* (polygonaceae) against human bacterial pathogen *P. aeruginosa*

Variations in the sensitivity of the bacterial species tested on the different medicinal plant extracts might be because of differences in the strains employed in the research and perhaps local environmental factors that affect the potency of medicinal plants, such as temperature, rainfall, day length and soil characteristics may have differed between the plant samples used for each study (Praveen and Sharmishtha, 2012). Aqil et al. (2005) reported that, plant extract was an alternative method to control pathogenic microorganism and many components of plant products have been shown to be especially targeted against resistant pathogenic bacteria.

Identification of bacterial isolate

The present selected pathogenic bacterial isolate (B24) was identified by using 16s ribosomal RNA as following Figure 1

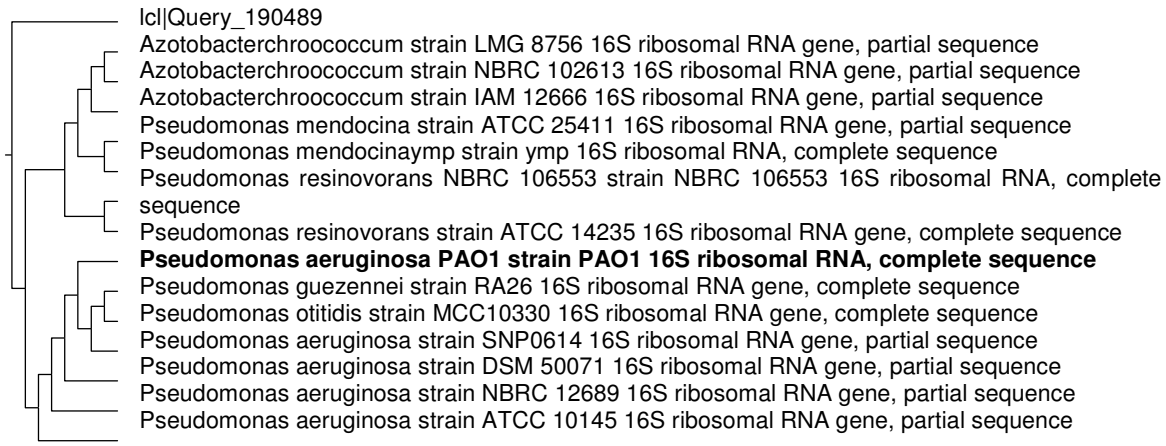


Figure 1. A dendogram showing the sequence relationships between *Pseudomonas aeruginosa* strain PAO1 0748281, B24 using 16S ribosomal RNA, complete sequence, and several other pathogenic strains based on 16s r RNA.

Table 1. Effect of different plant extracts on *P.aeruginosa* PAO1, B24.

Plant extracts	Concentrations(mg/ml)		
	100	**50**	**25**
Forsskaoleatenacissima	18.7 ± 0.58	17.7 ± 0.58	0.00 ± 0.00
Minuartiacumberlandensis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Euphorbiahirta	21.00 ± 1.00	19.00 ± 0.00	17.00 ± 0.00
Gemellmahystrix	18.70 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
Polygonum plebeium	**22.00 ± 0.00**	**25.3 ± 0.58**	**23.00 ± 0.00**
Kalanchoemarmorata	16.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Silenerubella	18.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
Thymusdecussatus	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Loliumtemulentum	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Antimicrobial activity of 9 medicinal plants against bacterial strain under study

The production of herbal drug preparations (i.e. extracts), using a variety of methods from simple traditional technologies to advanced extraction techniques is the first step in the value addition of medicinal and aromatic plants bioresources (Hanada et al., 2008).

In the present work from table (1) it was shown that, plant extract *P. plebeium*, PP at concentration 50 mg/ml was more effective against *P. aeruginosa* PAO1, B24 (25.33 mm).On the other hand, no effect at any concentration of plant extracts, *Thymus decussatus* ,TD and *Lolium temulentum*, LT on strain under study.

Pseudomonas species are often encountered in hospital and clinical settings and are a major cause of nosocomial infections. *Pseudomonas aeruginosa* was the predominant organisms implicated in almost every second infectious keratitis case (Seal and Pleyer, 2007).

These results were in accordance with (El-Mahmood, 2009) who showed that, the antimicrobial activity of the leaves extract of *E. hirta* was effective against *Salmonella* with diameter of inhibition zone18 mm. These results were also in accordance with (Lorna et al., 2015) who showed that, extract of *E. hirta* inhibited the growth of *P.aeruginosae* which has a low antibiotic susceptibility.

El-Mahmood et al. (2008) showed that, the phyto-chemical screening of the crude extracts of *E. hirta* revealed the presence of some bioactive components. It contains tannins, phenolics, anthraquinones, saponins, flavonoids and alkaloids. These compounds have potentially significant application against human pathogens, including those that cause enteric infections.

It was found that plant extract *P.plebeium* at concentration of 100 mg/ml was effective against *E.cloacae* strain ATCC 13047, B45 and *S.enterica* subsp. Houtenae, strain DSM 9221, B33 which inhibited the growth of bacterium by 25.66 mm and 20.33mm respectively. However, no effect at any concentration of *Thymus*

Table 2a. Determination of (MIC) of the plant extracts of *P.plebeium* against the growth of *P.aeruginosa* PAO1,B24

Bacterial strain	Plant extracts (mg/ml)				
	Mean diameter of inhibition zone of *P.plebeium* (mm)				
	100	50	25	12.5	6.2
P.aeruginosa, B24	21.67 ± 0 .58	24.67 ± 0.58	22.30 ± 0.60	20.33 ± 0.58	0.00 ± 0.00

Table 2b. Determination of (MIC) of the plant extracts of *E.hirta* against the growth of *P.aeruginosa* PAO1, no 0748281, B24

Bacterial strains	Plant extracts (mg/ml))				
	Mean diameter of inhibition zone of *E. hirta* (mm)				
	100	50	25	12.5	6.2
P.aeruginosa, B24	20.67 ± 0.58	21.67 ± 0.58	18.0 ± 1	14.33 ± 0.58	00.0 ± 00.0

decussatus and *Lolium temulentum* on *E.cloacae* strain ATCC 13047, B45. While, *L.temulentum* and *F.tenacissima* had no effect at any concentration on *S.enterica*, B33. (Sidkey et al., 2018)

It was shown that, plant extract *P. plebeium* , PP at concentration 100 mg/ml was the most medicinal plant effective against *K.pneumoniae* sub sp. pneumoniae HS11286 strain HS11286 (28.33 mm) but at concentration 50 mg/ml, it was more effective against *P.aeruginosa*, B24 (25.33 mm). Moreover, *E. hirta* ,EH at concentration 100 mg/ml was the most medicinal plant effective against *E.coli*, B42 (20.33 mm). On the other hand, no effect at any concentration of plant extracts, *Thymus decussatus*, TD and *Lolium temulentum*, LT on 3 strains under study (Desouky et al., 2018).

Kuta et al., 2013 showed that, the resistance by *K. pneumoniae* against the crude extracts of some medicinal plants could be due to the polysaccharide based capsule surrounding the cell of the organism.

Determination of MIC of *P. plebeium* and *E. hirta* against the growth of bacterial strain under study

Data illustrated in table (2a, b) declared that, the minimum inhibitory concentration (MIC) of plant extract *P.plebeium* and *E. hirta* against the growth of *P.aeruginosa* PAO1 strain PAO1, B24 was 12.5mg/ml.

These results agreed with (Lansing et al., 2005) whom reported that, MIC values of *P. aeruginosae* were low this occur due to low amount of active compounds contained in the crude extract.

Kuta et al. (2013) reported that, the minimum inhibitory concentration MIC of the aqueous, ethanol and methanol extracts of *E. hirta* against *K. pneumoniae, P.aeruginosa* and *Proteus vulgaris* ranged from 60mg /ml-100mg /ml.

These results were in contrast with (El-Mahmood, 2009) who indicated that, the minimum inhibitory concentration (MIC) of leaf extract *E.hirta* against the growth of *S.enterica* subsp. Houtenae, strain DSM 9221, B33 was 100mg/ml.

The minimum inhibitory concentration (MIC) of plant extract *P.plebeium* and *E. hirta* against the growth of *S.enterica* subsp. Houtenae, strain DSM 9221, B33 and *E.cloacae* strain ATCC 13047, B45 were 25mg/ml (Sidkey et al., 2018).

The minimum inhibitory concentration (MIC) of plant extract *P.plebeium* and *E. hirta* against the growth of *K. pneumoniae* subsp. pneumoniae HS11286 strain HS11286, *E.coli* O157:H7 str, B42. Sakai strain Sakai, and *P. aeruginosa* PAO1 strain PAO1, B24. MIC of plant extract *P. plebeium* against the growth of *P.aeruginosa* PAO1 strain PAO1, B24 was 12.5mg/ml whereas for *K. pneumoniae* subsp. pneumoniae HS11286 strain HS11286 it was 25mg/ml. On the other hand the (MIC) of plant extract *P. plebeium* against the growth of *E.coli* O157:H7 strain, B42. Sakai strain Sakai was 50 mg/ml . Moreover the data declared that, (MIC) of plant extract *E. hirta* against the growth of *P.aeruginosa* PAO1 strain PAO1, B24 was 12.5mg/ml and 50 mg/ml was more effective against the growth of *K. pneumoniae* subsp. pneumoniae HS11286 strain HS11286 and *E.coli* O157:H7 strain, B42. Sakai strain Sakai. (Desouky et al., 2018). Table 2a,b

Extraction of antimicrobial substances from *P. plebeium* and *E. hirta* plant extracts by different solvent systems

It was indicated from table (3) that, among all the tested

Table 3. Effect of different solvents of plant extracts *P.plebeium* and *E.hirta* on the selected pathogenic bacterial strain

Plant extracts	*Euphorbiahirta*			*Polygonum plebeium*		
Strain	n-hexan	ethyl acetate	n-butanol	n-hexan	ethyl acetate	n-butanol
P.earuginosa, B24	15.3 ± 0.58	32.00 ± 0.00	24.60 ± 0.58	20.00 ± 0.00	29.50 ± 0.50	25.00 ± 0.00

Table 4. Effect of ethyl acetate of (*P.plebeium*, PP and *E.hirta*, EH) and n-butanol of (*P.plebeium*, PP and *E.hirta*, EH) on the selected pathogenic strain

Strain	Ethyl acetate of (EH+PP)	n-butanol of (EH +PP)
P.aeruginosa, B24	32.00 ± 0.00	21.33 ± 0.57

solvent systems (n -hexane , ethyl acetate and n- butanol for the extraction of the antimicrobial agent from *P.plebeium* and *E.hirta* ethyl acetate was the best solvent for extraction of the antimicrobial substance of 2 medicinal plants. The synergistic effect was studied (table 4) indicated that, the mixture of (ethyl acetate) of both plant extracts of *E.hirta* and *P. plebeium* exhibited the highest antimicrobial activity against the bacterial strain under study.

Cooper and Kronenberg (2009) reported that methods used in the extraction of the plant material can influence the chemical composition of the extracts and potentially the biological activity is not new. The more information on the product that is provided in research, the greater will be the ability to compare among studies and understand differences in results that may emerge.

There is a clear indication that the solvent system plays a significant role in the solubility of the active ingredients in the plant and influences the antibacterial activities. This can be explained in terms of the polarity of the compound being extracted by each solvent and in addition to their intrinsic bioactivity, their ability to dissolve or diffuse in the media used in the assay (Ergene et al., 2006).

The results are in contrast to(Nikunj and Kaushik, 2014) who showed that, the acetone extract of *Euphorbia hirta* plant displayed an excellent antibacterial activity against *K. pneumoniae* but a methanolic extract of *E.hirta* plant displayed excellent antibacterial activity against *K. pneumoniae* and *P. aeruginosa* .

Among all the tested solvent systems n -hexane (F1), ethyl acetate (F2), and n- butanol (F3) for the extraction of the antimicrobial agent from *P.plebeium* and *E.hirta,* ethyl acetate (F2) was the best solvent for extraction of the antimicrobial substance of the 2 medicinal plants. The mixture of F2 (ethyl acetate) of both plant extract of *E.hirta* and *P. plebeium* exhibited the highest antimicrobial activity (Desouky et al., 2018)

Parameters controlling the growth of the selected strain in relation to mixed ethyl acetate extract of *E. hirta* and *P. plebeium*

For the purpose of improving the activity of mixed ethyl acetate extract of *P.plebeium* and *E.hirta* as an antimicrobial agent, different parameters were studied. Results recorded in Table (5) showed that, the best incubation temperature used for storage of the mixed ethyl acetate extract of *E.hirta* and *P. plebeium* was 45°C against *P.aeruginosa* PAO1, no 0748281, B24.It has been noticed that, the incubation time at such temperature plays an important role in the antimicrobial activity of the extract but on time up to it the antimicrobial activity was reduced.

The effect of temperature for methanolic extract of *E.hirta*, *K.pneumoniae* gave a zone of inhibition with diameter of 16mm at 100°C and 13 mm at 10°C. For aqueous extract, *K. pneumoniae* gave inhibition zone with diameter of 17 mm at 100°C and 13 mm at 10°C. Moreover, the diameters of zones of inhibition of hexane extract for *K.pneumoniae* 13 mm at 100°C and 9mm at 10°C (El-Mahmood, 2009).

According to (Sidkey et al., 2018) the best incubation temperature used for the storage of ethyl acetate extract of *E. hirta* and *P. plebeium* was 35°C against *E.cloacae* strain ATCC 13047, B45 but 25°C exhibited the higher antimicrobial activity against *S.enterica*.

The effect of different pH's on the antimicrobial activity of ethyl acetate extract of *P.plebeium* and *Ehirta* as an antimicrobial agent, against strain under study was tested, the results revealed that pH 5 was more effective against *P.aeruginosa* PAO1 no 0748281,B24 (24.7 mm) but on time progress it was less effective.

El-Mahmood (2009) reported that, acid stability is an important property of drugs, because it means that the plant components can be formulated to be taken orally and will not inactivate under the acidic conditions of the stomach and the gastrointestinal tract.

Table 5. Antimicrobial activity of plant extracts against *P.aeruginosa* PAO1, B24 in relation to different incubation temperatures

Time (hr)	Mean diameter of inhibition zone (mm)					
	Temperature °C					
	10	25	35	45	100	121
Zero	21.33 ± 0.57	27.33 ± 0.58	23.67 ± 0.58	22.0 ± 1.00	21.00 ± 0.00	
10min	20.33 ± 0.57	26.00 ± 0.00	24.00 ± 0.00	23.33 ± 0.58	22.67 ± 0.58	21.67 ± 0.58
1	18.67 ± 0.57	24.67 ± 0.58	26.33 ± 0.58	26.33 ± 0.58	20.00 ± 0.00	
6	18.33 ± 0.57	22.00 ± 0.00	24.33 ± 0.58	**29.0 ± 0.00**	19.67 ± 0.58	
24	18.0 ± 1.00	19.33 ± 1.53	22.33 ± 0.58	21.00 ± 1.00	18.60 ± 0.58	
48	17.0 ± 0.00	18.00 ± 0.00	20.00 ± 0.00	20.33 ± 0.58	0.00 ± 0.00	

Table 6. Antimicrobial activity of plant extracts against *P.aeruginosa* strain PAO1, B24 in relation to different pH's

Time (hr)	Mean diameter of inhibition zone (mm)					
	pH's					
	2	3	5	7	8	9
Zero	21.00 ± 0.00	21.33 ± 0.58	23.00 ± 0.00	24.67 ± 0.58	20.33 ± 0.57	18.33 ± 0.58
10min	21.67 ± 0.58	21.33 ± 0.58	**24.67 ± 0.58**	24.00 ± 0.00	21.67 ± 0.57	19.00 ± 1.00
1	20.00 ± 0.00	21.33 ± 0.58	21.33 ± 0.58	23.00 ± 0.00	20.0 ± 0.00	18.33 ± 2.08
6	19.67 ± 0.58	20.67 ± 1.15	21.0 ± 0.00	22.00 ± 0.00	19.33 ± 0.57	18.33 ± 0.58
24	19.00 ± 0.00	19.67 ± 1.15	20.00 ± 1.00	20.67 ± 0.58	18.33 ± 0.57	17.67 ± 0.58
48	18.33 ± 0.58	19.00 ± 1.00	19.33 ± 0.58	20.00 ± 0.00	18.00 ± 0.00	17.00 ± 1.00

El-Mahmood (2009) also indicated that, for aqueous extracts of *E. hirta* the zone diameters are 22 mm at pH 2 and 20 mm at pH10 for *K.* pneumoniae. A similar growth inhibition was exhibited by methanolic extract, the zone diameters are 25mm at pH 2and 23 mm at pH 10, growth inhibition by hexane extracts the zone diameters are 16 mm at pH 2 and 14mm at pH10.

The results obtained by (Desouky et al., 2018) on the antimicrobial activity of ethyl acetate extract of *P.plebeium* and *Ehirta* as an antimicrobial agent revealed that pH 5 was more effective against *K.pneumoniae* subsp. pneumoniae HS11286 strain HS11286, B38 (25 mm) and *P.aeruginosa* PAO1 strain PAO1, B24 (24.7 mm). On the other hand, pH 7 was effective against *E.coli* O157:H7 strain Sakai, B42 (24 mm) but on time progress it was less effective. Whereas (Sidkey et al., 2018) found that pH 7was more effective against *E.cloacae* strain ATCC 13047, B45 and *S.enterica* subsp. Houtenae, strain DSM 9221, B33 with mean diameter of inhibition zone were (25.0 and 22.3 mm) respectively. Table 6

In a trial to study the antimicrobial activity of ethyl acetate extract of *E.hirta* and *P. plebeium,* when it was subjected to light or dark for 24hr figure (2), it was found that the dark condition showed activation in the antimicrobial against *P.aeruginosa* PAO1, B24.

Concerning the effect of dark and light on ethyl acetate extract of *P.plebeium* and *Ehirta,* as an antimicrobial agent, light showed more activation in the antimicrobial activity compared with dark against *S.enterica* subsp. Houtenae, strain DSM 9221, B33 but against *E.cloacae* strain ATCC 13047, B45 dark treatment was more effective (Sidkey et al., 2018). Also (Desouky et al., 2018) found that the light condition was more effective against *E.coli* O157:H7 strain Saka, B42 and *K.pneumoniae* subsp. pneumoniae HS11286, B38. On the other hand, dark condition showed activation against *P.aeruginosa* PAO1 strain PAO, B24.

Also, it was found that, the antimicrobial activity of ethyl acetate of *E.hirta* and *P.plebeium* exhibited slight effectiveness under static condition, than dark, against *P.aeruginosa* PAO1, B24 with mean diameter of inhibition zone (28.67 mm) (figure 3).

The antimicrobial activity of ethyl acetate of *E.hirta* and *P.plebeium* exhibited slight effect under static condition against *E.coli* O157:H7 str. Sakai strain Saka, B42 and *P.aeruginosa* PAO1 strain PAO and the mean diameters of inhibition zones were (27.67 and 28.67 mm) respectively. While, under shaken condition at (50 rpm) it was effective against *K.pneumoniae* subsp. pneumoniae HS11286 strain HS11286,B38 (25.67 mm) (Desouky et al., 2018). In addition, the antimicrobial activity of ethyl acetate of *E.hirta* and *P.plebeium* exhibited slight effect under static condition against *S.enterica* subsp. with the

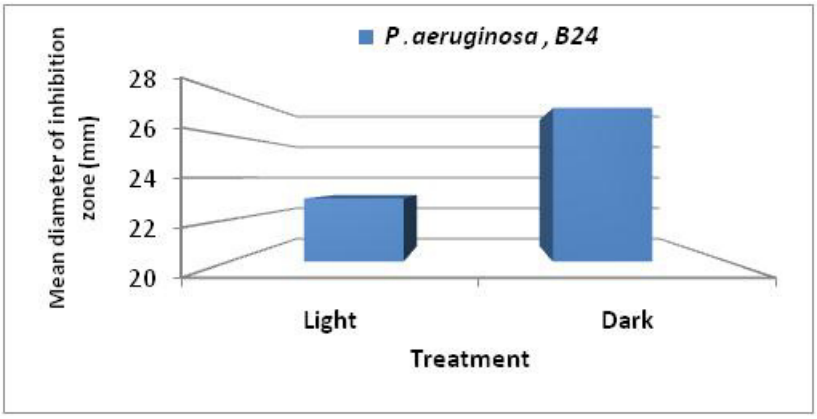


Figure 2. Antimicrobial activity of mixture of the ethyl acetate extracts of *P.plebeium* and *E.hirta* against *P.aeruginosa* PAO1, B24in relation to dark and light.

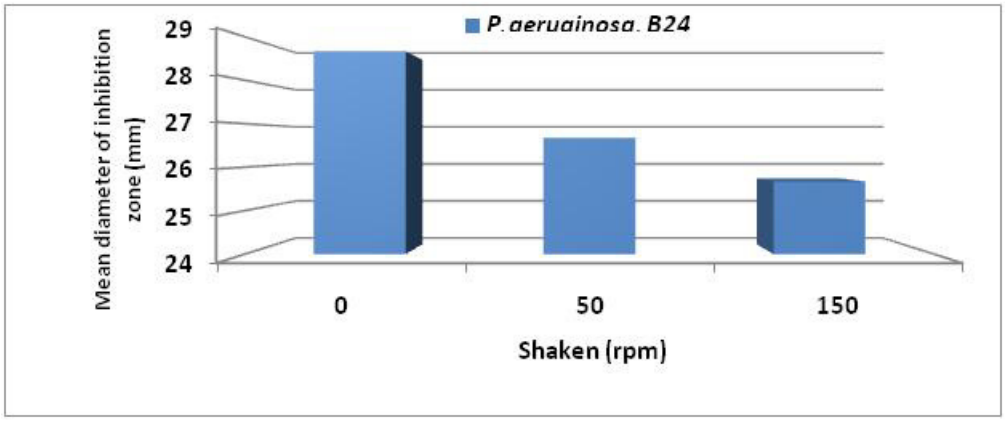


Figure 3. Antimicrobial activity of mixture of ethyl acetate extracts of *P.plebeium* and *E.hirta* against selected the strain in relation to static and shaken conditions

mean diameter of inhibition zone 27.33 mm. While, under shaken condition at (50 rpm) it was effective against *E.cloacae* strain (21.67 mm). This may be due to shaking could facilitate the release of the active substance in the water (Sidkey et al., 2018).

In the present investigation, on atrial to study the effect of introducing different carbon sources, in order to determine the best one which increased the antimicrobial activity of mixed ethyl acetate extract of *E.hirta* and *P. plebeium* against bacterial strain under study, it was found from figure (4) that, xylose was the best carbon sources which was more effective against, *P.aeruginosa* PAO1 (0748281), B24. This may induce synergitic activity with *E.hirta* and *P.plebeium* extract.

These results were in accordance with (Desouky et al., 2018) who found that xylose was the best carbon source effective against *K.pneumoniae* subsp. pneumoniae HS11286 strain HS11286, B38 and, *P.aeruginosa* PAO1 strain PAO1, B24. On the other hand, starch was more effective against *K.pneumoniae* subsp. pneumoniae HS11286 strain HS11286, B38. They may induce synergetic activity with *E.hirta* and *P.plebeium* extract. Also the monosaccharide xylose exhibited the highest antimicrobial activity in combination with ethyl acetate extracts of *P.plebeium* and *Ehirta* for microbial isolates *S.enterica* subsp. Houtenae, strain DSM 9221, B33 and *E.cloacae* strain ATCC 13047, B45 as stated by (Sidkey et al., 2018).

Concerning the effect of the addition of 12 different amino acids as nitrogen sources on the antimicrobial activity of mixed ethyl acetate of *E.hirta*, and *P. plebeium* against selected strain, Data from figure (5) declared that, no effect occurred by using different types of amino acids for the bacterial isolate under study.

On the other hand, L-phenyl alanine was the best amino acid which increased the antimicrobial activity of ethyl acetate extracts of *P. plebeium,* and *E. hirta* against *E.cloacae* strain ATCC 13047, B45 (Sidkey et al., 2018). Also, L-phenyl alanine was the best amino acid increasing the antimicrobial against *K.pneumoniae*

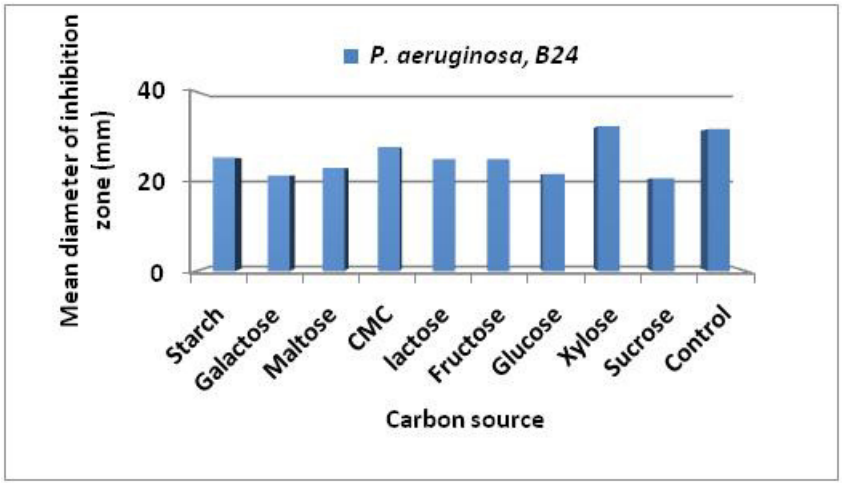


Figure 4. Effect of different carbon sources on the mixture of ethyl acetate extract of *P.plebeium* and *E.hirta* against selected pathogenic strain

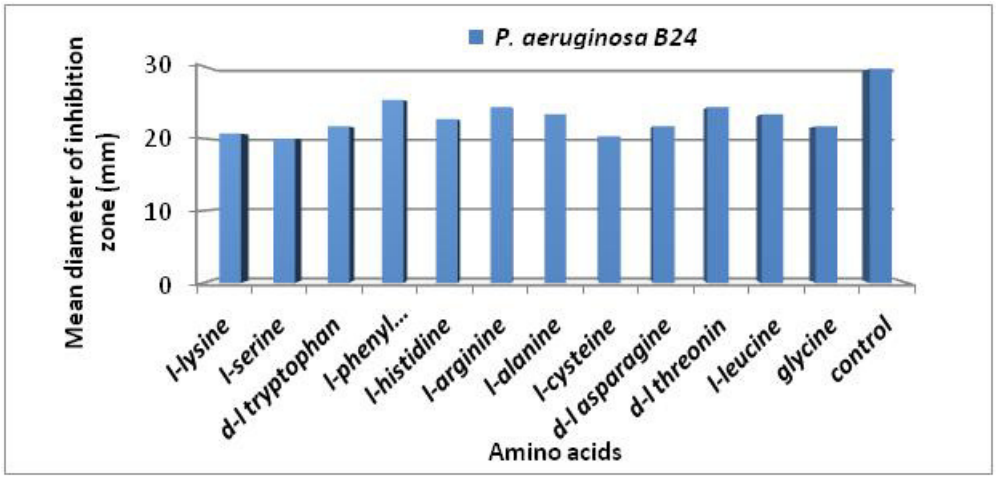


Figure 5. Antimicrobial activity of the mixture of ethyl acetate extract of *P.plebeium* and *E.hirta* against selected resistant strain in relation to different amino acids

subsp. nteric a HS11286 strain HS11286, B38 (24.67 mm) (Desouky et al., 2018).

By using different metallic ions with various concentrations (50,100, 200 ppm) in combination with mixed ethyl acetate extracts of *P.plebeium* and *E.hirta* against strain under study, it was found that, no metallic ions were effective as shown in figure (6).

(Desouky et al., 2018) stated that $CaCl_2$ with dilution 50 ppm when added to the ethyl acetate extracts of *P.plebeium* and *E.hirta* was effective, whereas, $MgSO_4$ with dilution 200 ppm was the best metallic ion for *K.pneumoniae* subsp. nteric a HS11286 strain HS11286, B38.

$CaCL_2$ at concentration (200 ppm) induced the highest antimicrobial activity against *E.cloacae* strain ATCC 13047, B45 (31.6 mm). On the other hand, $ZnSO_4$ at concentration (200 ppm) was the best metallic ion for *S.enterica* subsp. Houtenae, strain DSM 9221, B33(30.3 mm) as was stated by (Sidkey et al., 2018).

Data represented graphically in figure (7) indicated that, different vitamins with various concentrations in combination with mixedethyl acetate extracts of *P.plebeium* and *E.hirta* were studied. It was found that, no vitamins were effective on the bacterial strain under study. This in accordance with (Desouky et al., 2018) who studieddifferent vitamins with various concentrations in combination with ethyl acetate extracts of *P.plebeium* and *E.hirta*. It was found that, no vitamins were effective on *P.aeruginosa*.

On the other hand (Shaista et al., 2009) revealed that different vitamins with various concentrations in combination with ethyl acetate extracts of *P.plebeium* and

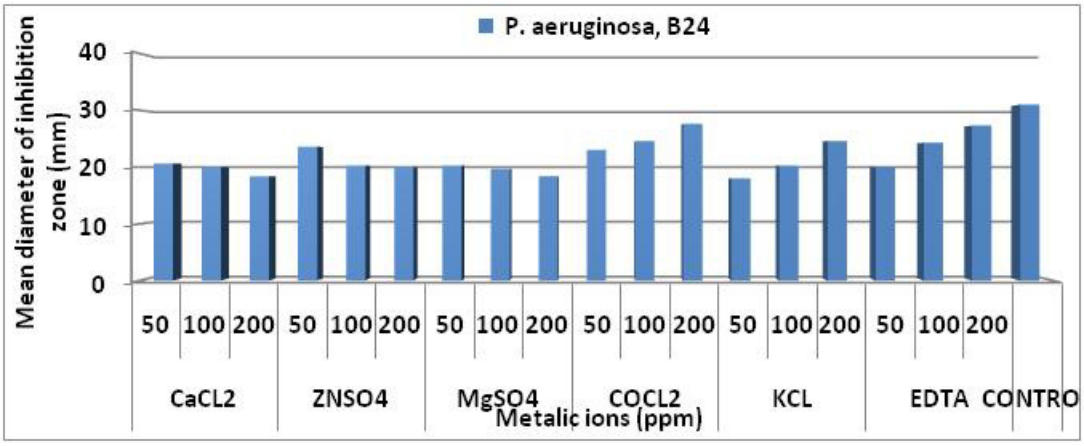


Figure 6. Antimicrobial activity of the mixture of ethyl acetate extract of *P.plebeium* and *E.hirta* against selected resistant strains in relation to different metallic ions

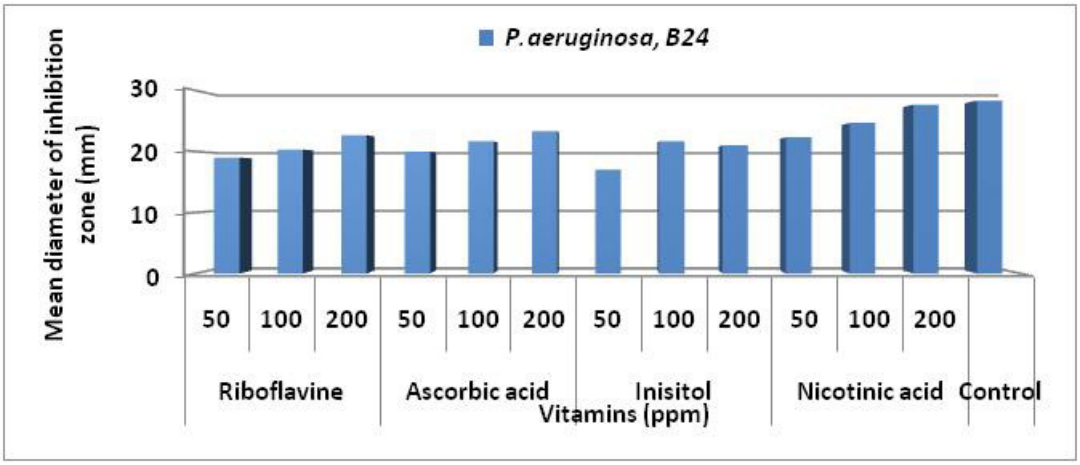


Figure 7. Antimicrobial activity of the mixture of ethyl acetate extract of *P.plebeium* and *E.hirta* against selected resistant strain in relation to different vitamins

E.hirta were studied and ascorbic acid was the best vitamin against *E.cloacae* strain ATCC 13047, B45 with dilution 100 ppm with mean diameter of inhibition zone 23.67 mm . This finding agrees with the fact that ascorbic acid had efficient antimicrobial properties. Ascorbic acid has been used to extend the shelf life of various fruits and vegetables (Sapers and Miller, 1995). These results were also in complete accordance with(Tsuchiya etal., 1996)who reported that, ascorbic acid may enhance or stabilize the flavonoids that are responsible to disrupt the microbial membrane.

Separation and purification of the antimicrobial substance from *E.hirta* and *P.plebeium* by Column chromatography

In view of the findings of other workers, column chromatography packed with silica gel and an eluting solvent composed of various ratios of solvent system was used for fractionation of the crude extract into active fractions (Tabata et al., 1997; Imnagaki et al., 1998; Momose et al., 2001; El-Henawy, 2006; El- Tantawy, 2008; Khalifa et al., 2008).

Column chromatography for separation of active antimicrobial substance from ethyl acetate extract of *P.plebeium*

Data illustrated graphically in Figure (8, 9) Indicated that, the maximum activity could be recorded in 2 peaks. Peak I fractions (43-53) inhibited the growth of *P. aeruginosa* PAO1 strain PAO, B24 by (15.0, 15.6, 17.6, 22.6, 27.3, 31.6, 37.6, 30.3, 20.6, 16.3 and 14.3 mm) respectively. Moreover, peak II fractions (58:63) inhibited

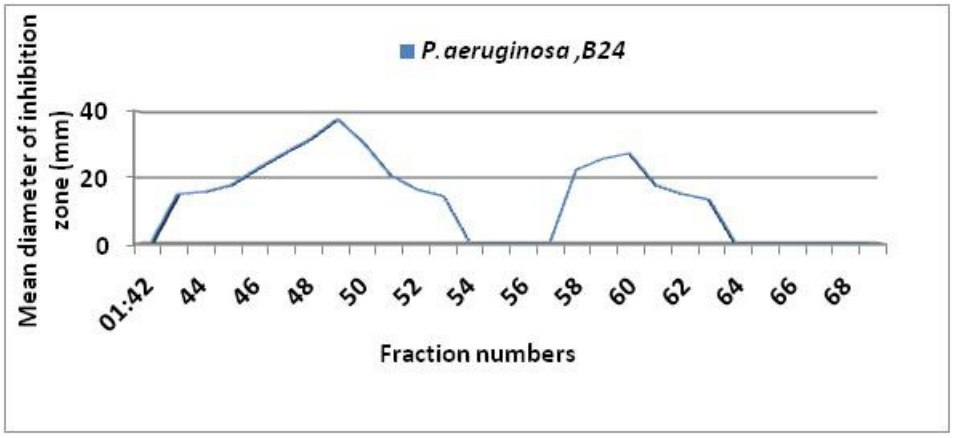


Figure 8. Fractionation pattern of active antimicrobial substance from *P.plebeium* extract and their effect against selected pathogenic strain

The clear zone on the left reffered to Peak I, and on the right reffered to Peak II

Figure 9. Fractionation pattern of active antimicrobial substance from *P.plebeium* extract and their effect against *P.aeruginosa* PAO1, B24

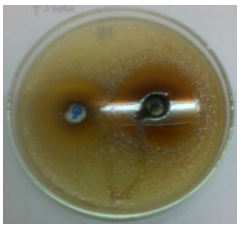
The clear zone on the left reffered to Peak II, and on the right reffered to Peak I

Figure 10. Fractionation pattern of active antimicrobial substance from *E.hirta* extract and their effect against *P. aeruginosa* PAO1 (0748281, B24

the growth of *P.aeruginosa* PAO1, B 24 by (22.3, 25.6, 27.3, 17.6, 15.0, 13.3 mm).

In view of the findings of other researchers, (Janakat et al., 2004) found that, the antimicrobial activity was within the third fraction of column chromatography. This fraction was then subjected to gel filtration using Sephadex G-100.Two peaks were obtained. Peak one possessed higher antimicrobial activity. Only peak 4 from six peaks obtained showed as a light antimicrobial activity.

Rios and Recio (2005). Showed that, the chloroform extracts exhibited the least antimicrobial activity as compared to other solvent extracts from different medicinal plants. The reasons could be a low concentration of antimicrobial compounds in these extracts.



The clear zone on the left referred to Peak I (PP) and PII (PP), and on the right referred to Peak I (PP) &PII (EH)

Figure 11. The antimicrobial activity of a mixture of peak I and peak II for *P.plebeium* (PP), and mixture of peak I of *P.plebeium*, PP and peak II of*E. hirta,* EH against *P. aeruginosa* PAO1, B24.

Column chromatography for separation of active antimicrobial substance from ethyl acetate extract of *E.hirta*

Data illustrated graphically in figure (10) Indicated that, Peak I fractions (53:60) inhibited the growth of *P.aeruginosa* PAO1, B24 by (14.6, 19.6, 21.6, 27.6, 23.6, 18.6, 14.3 and 13.6 mm) respectively. Moreover, peak II fractions (65:72) inhibited the growth of *P.aeruginosa* PAO1, B 24 by (24.3, 28.3, 30.6, 27.3, 21.3, 17.6, 14.6 and 13.3 mm).So Peak II of *E.hirta* exhibited the highest antimicrobial activity with strain under study.

In atrial to elucidate the synergistic effect of mixture of all active peaks, the active fractions in each peak were mixed together in glass vials, and then were used for the antimicrobial activity test against the strain under study. Data in figure (11) showed that, The mixture of peak I of *P .plebeium* and peak II of *E.hirta* was the most effective against the strain under study (*P.aeruginosa* PAO1, B 24 by (38.0 mm) .

Characterization of the active compounds as antimicrobial agents from *E.hirta*

The number of studies demonstrating the use of infrared spectroscopy in combination with chemometrics for analyses of complex samples has grown dramatically. The main advantages are its speed, environmental friendliness, price, and possibility of in-line automatization since no or easy sample preparation is needed, and spectral acquisition is simple and fast once a good chemometric model has been developed. FT-IR has been used to determine flavonoids in different plant leaf samples and quercetin glycosides inonion (Allium cepa). https://www.researchgate.net/publication/318884282_FT-IR based_method_for_rutin_quercetin_and_quercitrin_quantification_in_different_buckwheat_Fagopyrum_species [accessed Jun 23 2020].

Aliphatic hydrocarbons exist in simple linear chains, branched chains and in cyclic structures. Any one molecule may exist with one or more of these structures. The infrared spectrum can provide information on the existence of most of these structures, either directly or by inference. The introduction of unsaturation in the form of a double or triple bond has a profound impact on the chemistry of the molecule, and likewise it has a significant influence on the infrared spectrum. Infra-red spectrum provides information specific to the group itself, and also on the interaction of the group with other parts of the molecule and on the spatial properties of the group. Examples include conjugation between a double bond and another unsaturated center, an aromatic ring or a group, and the orientation or location of the double bond within the molecule, spectral contributions are characterized, as CH stretching and bending vibrations and CC vibrations (stretching and bending), which for the most part is unique for each molecule, and are generally described as skeletal vibrations. Likewise, the same can be said for the unsaturated carbon–carbon multiple bonding in alkene and alkyne (INTERPRETATION OF INFRARED SPECTRA, A PRACTICAL APPROACH, A Practical Approach John Coates Consulting, Newtown, USA. Encyclopedia of Analytical Chemistry R.A. Meyers (Ed.) Copyright John Wiley & Sons Ltd).

The most active fractions on the 6 strains under study were collected (53-54-55-56- 58-59-62-63-67-68-69). The purified fractions were checked by chromatographic separation on silica gel TLC plate that showed two bands under short wavelength. Also, the purity of this compound was confirmed by the total ion chromatogram resulted from mass spectroscopy (MS) analysis of 2 substances.

Characterization of the active compound (A) from *E.hirta*

The purity of the compound was confirmed by the total

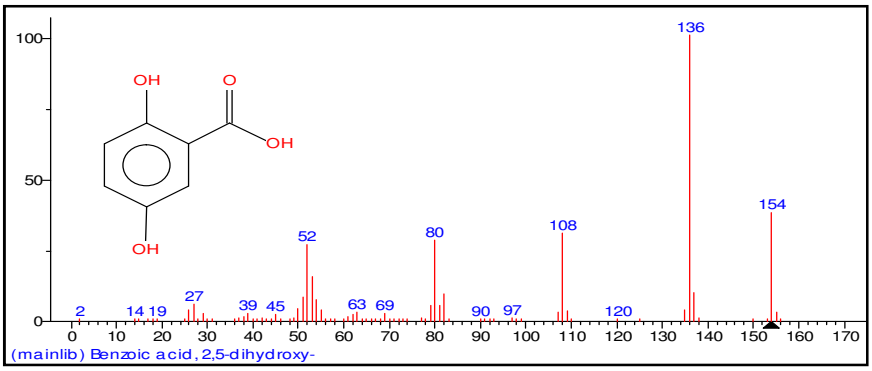


Figure 12. The mass spectroscopy of compound (A) from *E. hitra*

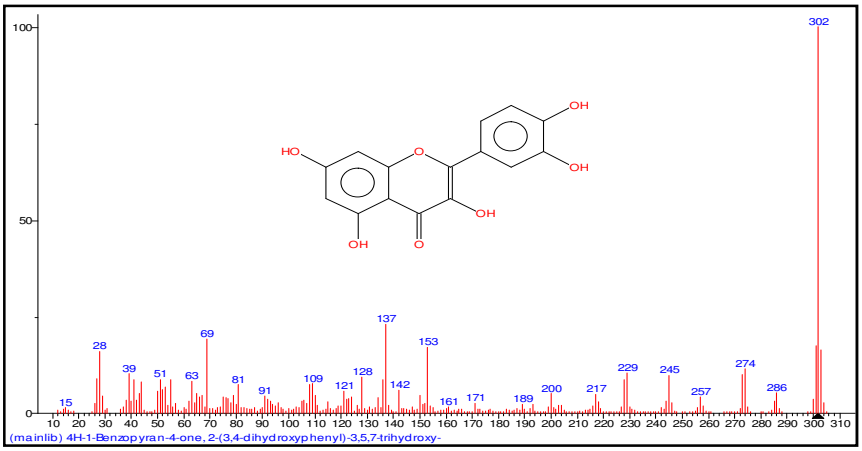


Figure 13. The mass spectroscopy of compound (B) from *E.hitra*

ion chromatogram resulted from mass spectroscopy (MS) analysis and the mass spectrum showed molecular ion peak at m/z 145 (30%), 108 (29%), 80 (26%), 52 (25%), 27(11%) and the base peak at m/z 136 (100%).The molecular formula for the compound is $C_7H_6O_4$ and the chemical name is gentesic acid (or benzoic acid) (2, 5 dihydroxybenzoic acid or 5- hydroxysalicylic acid) as shown in figure (12). Gentesic acid (or benzoic acid) was isolated as yellowish white powder, gave blue color with ferric chloride indicate it is a phenolic in nature. Ultra violet (UV) spectrum of this compound ƛmax MeOH nm (log3): 248, 310; Infrared (IR) spectrum nmax film cm1: 3350–3515 (broad), 1615, 1600, 1475.

The characteristic bands of the COOHgroup near 1700, 1300 and 950 cm.-1 were observed as pairs.This appears to indicate that two kinds of configurations having different energies and different spectra exist in the crystal. The energy difference was estimated to be about 0.1 Kcal. /mole from measurements of intensity ratios of pairs at various temperatures. The nearly equal distances of the two C—O bonds in benzoic acid, i.e. 1.29 and 1.24 A as determined by X-ray measurements, were interpreted as the average for the mixture of the two configurations**.**

There are two bands at 1706 and 1684 cm^{-1}in the region of the stretching vibration of C=0 bonds of the dimeric units. The band at 1432cm^{-1} or 1421 cm^{-1} at room temperature is due to one of the coupled vibrations of C—O stretching and OH bending, the two bands at 1334 and 1298 cm^{-1}, or 1324 and 1288 cm^{-1} at room temperature, respectively, are in the region of another coupled vibration of C—O stretching and OH bending, and those at 959 and 948 cm^{-1}.

Characterization of the active compound (B) from *E. hirta*

The purity of the compound was confirmed by the total ion chromatogram resulted from mass spectroscopy (MS) analysis and the mass spectrum showed molecular ion peak at m/z 274 (10%), 153 (10%), 137 (18%) and base peak at m/z 302 (100%). The molecular formula for the compound is $C_{15}H_{10}O_7$ and the chemical name is quercetin as shown in figure (13). Quercetin was yellow crystals. Ultra violet (UV) spectrum of this compound λMax nm in MeOH: 370, 298(sh), 256; NaOMe 423, 302(sh), 260 (dec); AlCl3 446, 292(sh), 270; AlCl3/HCl

424,360,290(sh), 270; NaOAc 360, 292(sh), 270; NaOAc/ H3BO3 380, 299 (sh), 260.

The chemical constituents of *E.hirta* are gallic acid, quercetin and phenolic substance. These substances are responsible for its medicinal properties. The plant can be boiled and drank to expel worms *E.hirta* possesses antibacterial, anthelmintic antifungal and antimalarial properties (Lorna et al., 2015).

Abhinav et al. (2014) reported that, Quercitrin, a flavonoid and glycoside isolated from *E. hirta* showed an antibacterial activity and have m.w:302.238 g/mol.

CONCLUSION

This study investigated the effect of the leaf extracts of *Euphorbia hirta* and *Polygonum plebeium* against human bacterial pathogen *Pseudomonas aeruginosa* isolated from wound infection in Cleopatra Hospital. Among all tested solvents, ethyl acetate was the most effective. Also the ethyl acetate extract of both medicinal plants had synergistic effect which increased the antimicrobial activity against *P. aeruginosa*. MIC was found to be 12.5mg/m. Parameters controlling the antimicrobial activity was studied. It was more effective when the extract was incubated at 45°C, pH5, dark condition for 24hragainst *P. aeruginosa*, static condition had slight effect. Xylose exhibited the highest antimicrobial activity among other carbon sources. Different types of amino acids, metallic ions and vitamins were not effective on the microbial strain under study. Since the synthetic drugs are more costly and lead to side effects, while the usage of herbal medicines show promising results and less or no side effects, so active biological compounds, prepared from various parts of medicinal plants, can be used as antimicrobial agents alternative to the chemical synthetic ones.

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