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The Study of Antibacterial Effects of Combining Some Medicinal Plants with Certain Antibiotics Against the Multidrug-Resistant *E. coli*.

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ABSTRACT

As resistant microorganisms are causing a serious global problem, medicinal plants had begun to draw scientists' attention to use them as alternative medicines or in case of this research; to be combined with antibiotics to find a solution against one of these resistant microorganisms. This research shows the possibility of getting positive results in inhibiting the growth of Multidrug-resistant *E. coli*; through the synergism between medicinal plant extracts with antibiotics. Each of the alcoholic and aqueous extract of the plants: *Glycyrrhiza glabra*, *Viscum album* and *Chamaemelum nobile* were combined with Erythromycin ethylsuccinate and Gramicidin in certain percentages individually to get three types of combinations (A, B and C). Combinations that contained more percentages of extracts than antibiotic in them showed the highest synergistic positive results in inhibiting the growth of *E. coli*. Furthermore, these plant extracts as the phytochemical screening tests showed, contain certain active contents which in rule have antimicrobial effects.

INTRODUCTION

New resistance mechanisms of some microorganisms against antimicrobials are emerging and spreading globally (WHO, 2018). Resistance is caused by the misuse and overuse of antibiotics; which encourages bacteria to develop new ways to overcome their effects (Summers,2008). In addition, antibiotics might possess adverse effects in the body, which include hypersensitivity, immunosuppressant and allergic reactions (Singh et, al.,2010; Kumar and Singh,2013). Thereby threatening our ability to treat common infectious diseases, resulting in prolonged illness, disability, and death (WHO, 2018).

These situations forced scientists to explore for new antimicrobials from various sources, such as medicinal plants (Karaman, 2003).

For a long time licorice (***Glycyrrhiza glabra***) and Mistletoe (***Viscum album***) have been used throughout the world as a drug and a remedy for various diseases (Kaegi, 1998; Tian et, al 2008). Roman chamomile (***Chamaemelum nobile***) is not a new used herb either; it started to be cultivated in Rome in the 16th century to be used in medicine and as a consuming substance (Chevallier,2016).

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Chamomile (***Chamaemelum nobile***) possesses antibacterial, antifungal, insecticidal, hypotensive, anti-platelet aggregation, anti-inflammatory, hypoglycaemic, antioxidant, nervous, cytotoxic, bronchodilator, endocrine and many other effects (Al- Snafi, 2016). Licorice (***Glycyrrhiza glabra***) as well, possesses Antioxidant activity (Visavadiya et, al. 2009), anti-inflammatory activity (Baker, 1994), anti-viral effects (Alonso,2004) anti-fungal activity (Hojo and Sato, 2002), anti-bacterial Activity (Sharma et, al. 2013).

European mistletoe (***Viscum album***) is also used for managing wide range of diseases, such as chronic cramps, stroke, stomach problems, breathing difficulties, according to many folk medicine traditions (Ohiri,2003). Hypotensive, vasodilator, cardiac depressive, sedative, antispasmodic, anticancer, and antidiabetic effects of ***Viscum album*** have been demonstrated in previous studies (Yesilada, 1998; Gray and Flat, 1999; Orhan et, al. 2005).

Phytochemicals such as flavonoids have showed antimicrobial (Vasudevan *et al*., 2013), anti-inflammatory, antiallergenic, anti-viral, and anti-carcinogenic activity (Cody *et al.,* 1986). Saponins also posesses anti-inflammatory, anti-microbial, anti-protozoan, and immunostimulatory properties (Francis *et al*., 2002; Sparg *et al*., 2004). Triterpens might contribute in antbacterial activity (Arruda *et al.*, 2011) and also helping in inflammatory response (Dudhgaonkar *et al*., 2009). Plant Phytosterols also play a role in the immune system functionalism. They possess an effect on lymphocyte activity (Wasserman, 2011; Bouic, 2001). Carbohydrates such as honey possesses a huge amount of health beneficial properties beginning with bactericidal and antiseptic properties , to serious conditions such as urinary disorders, pulmonary tuberculosis. Glycosides like saponin glycosides which are found in licorice possesses anti-inflammatory effects. Another example is phenol glycosides which are found in Bearberry and has a urinary antiseptic effect (Shah and Seth, 2010). Alkaoids may have antimicrobial activities (Harborne, 1993). Polyphenols (phenolic acid, tannins, isoflavones green tea catechins) have been reported to inhibit the reproduction and growth of many fungi, yeasts, viruses and bacteria, such as Salmonella, Enterotoxigenic *E. coli* (ETEC) (Howell, 2007).

Combined therapy is traditionally used to increase antimicrobial activity and reduce toxic effects of agents (Houghton, 2009). Combination has numerous benefits that include treatment of mixed infections and infections caused by specific causative organism; to increase antimicrobial activity preventing need for long term antibiotic use and prevent emergence of multidrug resistant bacteria (Levinson and Jawetz, 2002). The combination of two drugs can be synergistic, additive or antagonistic (Nascimento *et al.,* 2000). Synergism has been defined as phenomena in which two different compounds are combined to enhance their individual activity. If the combination results in worsening effect, it is called antagonism; effect which is less than synergistic but not antagonistic is termed as additive or indifference (Rani *et al.,* 2009).

MATERIALS AND METHODS

Obtaining *E. coli* Sample:

E. coli has been obtained as pure cultures from the University of Sulaimani. They were identified by the regular procedures mentioned in (Bridson, 2006; Wise,2017).

Plant Samples:

All plant samples (*Glycyrrhiza glabra*, *Viscum album* and *Chamaemelum nobile*) were obtained from Iraqi herbariums in Baghdad and Salahuddeen Governorates. They were identified and classified by a specialized professor (Dr. Shakir Mahdi Salih in University of Tikrit - College of Agriculture).

Preparation of Plant Extracts and Phytochemical Screening:

All plants were cleaned, air dried, powdered and were kept till the time of using. The parts of plants that were used were; Flowers from *Chamaemelum nobile*, Fruits/berries– from *Viscum album* and roots from *Glycyrrhiza glabra*.

Extracting:

Extraction was carried out by the method described in (Mythili and Ravindhran**,** 2012). Ethanol and water were used individually as solvents. The extracts were obtained through the cold percolation method. The powdered plant materials were weighed and then soaked in absolute ethanol and water individually for 72 hours (100gm of each plant was soaked in 500 ml of each solvent). Then the extracts were taken by filtering the content. The same procedure was repeated again and the extracts were collected for each of the solvents. The extracts were pooled together and concentrated on a water bath by keeping the temperature below the boiling point of the solvents that were used (78◦ C for ethanol and 100◦C for the water). The concentrated extracts were kept in the desiccator for further evaporation of the solvents. Then the extracts were weighed and the yield was recorded.

Screening for Phytochemicals in Plants:

All the screening tests were carried out according to (Mir, et al.,2016; Fransworth, 1966), with slight modifications.

Quantitative Analysis of Total phenols:

The amount of total phenols in all extracts was determined according to the Folin-Ciocalteu procedure (Singleton et al , 1974). Samples from each plant extracts (2 mL, triplicates) were inserted into test tubes; 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added to them. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (Systronics UV-vis spectrophotometer). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

Quantitative Analysis of Alkaloids:

Preparation of Solutions:

Bromocresol green solution was prepared by heating 69.8 mg of bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until it was completely dissolved and the solution was diluted to 1000 ml with distilled water. Phosphate buffer solution (pH 4.7) was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na_2HPO_4 in 1 L distilled water) to 4.7 with 0.2 M Citric acid (42.02 g citric acid in 1 L distilled water). Caffeine standard solution was made by dissolving 1 mg pure caffeine in 10 ml of distilled water.

Preparation of Standard Curve:

Accurately aliquots (0.4, 0.6, 0.8, 1 and 1.2 ml) of caffeine standard solution was measured and transferred into several separatory funnels. Then, 5 ml of pH 4.7 phosphate buffer and 5 ml BCG (Bromocresol green) solution were added and then mixed with1, 2, 3 and 4 ml of chloroform, then the whole mixtures were shaken. The extracts were collected in a 10 ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without caffeine (Fazel et, al , 2008)

Preparing samples for Absorbance:

A part of each extract powders was dissolved in 2 N HCl and then filtered. One ml of this solution was transferred to a separatory funnel and washed with 10 ml chloroform (3 times). The pH of this solution was adjusted to neutral with 0.1 N NaOH. Then 5 ml of BCG solution and 5 ml of phosphate buffer were added to this solution. The mixture was shaken and the complex formed was extracted with 1, 2, 3, and 4 ml chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted with chloroform. The absorbance of the complex in chloroform was measured at 470 nm.

Preparation of Media:

Nutrient Agar [Oxoid]: It was prepared by dissolving 23g of the medium powder into 1 liter of distilled water. Then the pH of the medium was adjusted to 7.3 . The medium was autoclaved at 121◦c and 1 psi (Pound-force per Square Inch) for 15 minutes.

Nutrient Broth [Oxoid]: It was prepared by dissolving 13g of the medium into 1 liter of distilled water. Then its pH was adjusted as same as the previous medium and was autoclaved at the same temperature and pressure.

Medium 1 [Oxoid]: This medium was prepared according to the method described in the Oxoid reference, 27g of the medium powder was suspended in 1 liter of distilled water. Then it was boiled till the medium powder was completely dissolved and autoclaved at 121°c and 1 psi for 15 minutes (Bridson, 2006).

Maintenance of Microorganisms samples:

Bacterial samples were preserved in nutrient agar and activated by nutrient broth.

Antimicrobial Potency Assay:

Neomycin was used as a standard control with the plant extracts in all media plates. All antimicrobials were used according to the same reference.

According to Wise (2017) and Bridson (2006), the medium that was used in the "Cylinder-plate" method in order to carry out the antibiotic susceptibility test of standard antibiotics and plants extracts against *E. coli* was (medium 1). Different concentrations of each plant extract (250, 500 and 1000mg/ml) were taken to be tested for their antimicrobial effects against *E. coli* (Ismail, *et al*., 2013). While the concentration of all standard antibiotics was (0.01 mg/ml) according to the USP (Wise, 2017).

The test was carried out by inserting extract solutions or standard antimicrobials into the cylinders of the media, individually, and after incubation the inhibition zones were measured by the zone reader apparatus.

Preparing combinations of (antibiotic and extracts):

According to the procedure that was carried out in (Ismail, *et al*., 2013), three types of percentages (25, 50, and 75%) were made out of the aqueous extracts and the alcoholic extracts; and they were mixed with the opposite percentages of the standard antibiotics (Erythromycin ethylsuccinate and Gramicidin) individually to make 1 ml of each combination, as follows:

1- 0.25 ml (antibiotic) : 0.75 ml (extract).
2- 0.50 ml (antibiotic) : 0.50 ml (extract).
3- 0.75 ml (antibiotic) : 0.25 ml (extract).

The concentration of the aqueous extracts was 500mg/ml, while the concentration of alcoholic extracts was 250mg/ml. These concentrations were reduced from 1000 which was the maximum concentration; in order to prevent any interference among the inhibition zones, also to explore the antimicrobial effect of lesser concentrations when they are mixed with antibiotics. 60 µl of each combination were placed into the solidified agar cylinders (wells) to act as an antimicrobial in the cylinder plate method. All media plates were incubated then, all inhibition zones that appeared around the wells were measured by zone reader apparatus

The previous process was repeated with all the type of the plants extracts against the Multi-drug Resistant *E. coli*.

Statistical Analysis: Statistical analysis were carried out according to Duncan's Multiple Range Test at probability value of $p \leq 0.05$.

RESULTS AND DISCUSSIONS

Results of Phytochemical Screening:

As table 1 shows, phytochemical screenings for phenols, glycosides and flavonoids showed positive results in all of the plants extracts. Alkaloids were only present in the alcoholic extracts of the plants. The reason that alkaloids didn't appear in the aqueous extracts is because alkaloids dissolve

poorly in water (CIHR *et, al.*, 2018). Triterpenes and carbohydrates were detected in almost all of the extracts except for the aqueous extract of *Viscum album*. Saponins as well, were detected in all plant extracts except for the aqueous and alcoholic extracts of *Viscum album*. The waxy nature of the *Viscum album* berries might have affected the detection of some phytochemicals in it (Kewscience, 2018). Sterols were detected only in alcoholic extracts of all plants but, didn't show positive results in all of the aqueous extracts, because they might have been insoluble in water; since sterols are amphipathic lipids (Fahy et, al.,2005). These results are shown in table 1.

Results of Total Phenolic and Alkaloid Content:

Table 2 shows that *Chamaemelum nobile* possessed the highest value of total phenols which was 64.85 mg/g that is significantly different than 51.87 in *Glycyrrhiza glabra*. Depending on the organ and the stage of growth since generally the total polyphenol content is higher in plants at the flowering stage than at the vegetative stage (Medini *et, al*., 2014). The parts that were used from *Chamaemelum nobile* were only the flowers.

Glycyrrhiza glabra exhibited the highest value of alkaloid content which was 1.58 mg/g, because unlike phenols, alkaloids are more involved in plant defense than attracting pollinating and seed spreading animals (Fattorusso and Tagliatella, 2008; wink, 2000), and the part that was used from *Glycyrrhiza glabra* was the roots, therefore it contains more alkaloids than *Viscum album* (berries) and *Chamaemelum nobile*.

Table 1: Phytochemical Screening Tests of the Plant Extracts.

Plants	Phytochemicals															
	Phenols		Alkaloids		Triterpines		Sterols		Saponins		Carbohydrates		Glycosides		Flavonoids	
	Aq.*	Al.*	Aq.	Al.	Aq.	Al.	Aq.	Al.	Aq.	Al.	Aq.	Al.	Aq.	Al.	Aq.	Al.
Chameamelum nobile	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Glycyrrhiza glabra	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Viscum album	+	+	-	+	-	+	-	+	-	-	+	-	+	+	+	+

- Aq.*= Aqueous extract, Al. * = Alcohol extract.
- (+ / -) = availability of the phytochemicals.

Table 2: Total Phenols and Alkaloids in Plant Extracts.

Plant Extracts	Total Phenols (mg/g)	Total Alkaloids (mg/g)
Chamaemelum nobile	64.85 A	0.093 C
Glycyrrhiza glabra	51.87 C	1.58 A
Viscum album	59.54 B	1.03 B

- Statistical analysis was carried out by Duncan's test, P value ≤ 0.05 .
- Different letters mean that there are significant differences among the results.

Standard Antibiotic Potency:

E. coli exhibited resistance against Erythromycin ethylsuccinate and Gramicidin, therefore they were used in the combination tests to explore the effects of combing them with the extracts against the multidrug-resistant *E. coli*. Neomycin possessed the highest efficacy in inhibiting the growth of *E. coli* (22 mm) which was significantly different than what the rest of the antibiotics have shown as the table 3 shows.

Table 3: Antibiotic Potency Against *E. coli*.

Bacteria	Inhibition Zones of Antibiotics Against The Bacterial Growth (mm).					
	Erythromycin ethylsuccinate	Gramicidin	Neomycin sulfate	Gentamicin sulfate	Tetracycline	Amoxicillin
E. coli	0 E	0 e	22 A	11 D	13 c	15 b

- According to the statistical analysis which was carried out by Duncan's test, the different letters means that there are significant differences among the results, while the same letters means the opposite.

Antibacterial Effects of the Plant Extracts:

E. coli was resistant to all of the alcoholic and aqueous extracts in all concentrations. A standard antibiotic was used as a control and showed inhibition zones of 22.3 cm. This is shown in Table 4.

The Combination of Antibiotics with Extracts Effects:

Table 5 shows that increasing the percentages of the plants extracts in the combinations A (75%) comparing to the percentages of the antibiotics (25%) in them tends to increase the inhibition zones relatively. In the first combinations (A), combining 75% of ***Chamaemelum nobile*** alcoholic extract with 25% of erythromycin showed an inhibition zone of 17.1 mm against the *E. coli* growth, whereas combining 75% of the same antibiotic with 25% of ***Chamaemelum nobile*** alcoholic extracts in the combinations (C) showed an inhibition zone of 10.8 mm; which shows a big significant difference between the two. This denotes that there was an enhanced synergism in the combination that contained the highest amount of plant extract. Also there is an enhanced synergism in combining the plant extracts with the inactive antibiotics, comparing to their individual effect against the resistant microorganism. The enhanced synergism was caused by adding or increasing concentrations of the plant extracts comparing to the antibiotic concentrations.

Table 4: Antibacterial Effects of the Plants Extracts Against *E. coli*.

Extract Types	Inhibition Zones of The Plant Extracts Against *E. coli* Growth (mm).											
	Chamaemelum nobile				*Glycyrrhiza glabra*				*Viscum album*			
	St.	250 mg/ml	500 mg/ml	1000 mg/ml	St.	250 mg/ml	500 mg/ml	1000 mg/ml	St.	250 mg/ml	500 mg/ml	1000 mg/ml
Alcoholic Extracts	22.3 aA	0 bB	0 bB	0	22.3 aA	0 bB	0 bB	0 bB	22.3 aA	0 bB	0 bB	0 bB
Aqueous Extracts	22.3 aA	0 bB	0 bB	0	22.3 aA	0 bB	0 bB	0 bB	22.3 aA	0 bB	0 bB	0 bB

- St. "Reference Standard USP" = Neomycin Sulfate (0.01 mg/ml).
- According to Duncan's test, the statistical analysis shows that the different letters mean that there are significant differences among the results, while the same letters mean the opposite

Table 5: The Combining Effects of Antibiotics with Plant Extracts against *E. coli* :

Antibiotics	Inhibition Zones of Combinations of Plants Extracts and Antibiotics Against *E. coli* Growth (mm).																	
	Chamaemelum nobile						*Glycyrrhiza glabra*						*Viscum album*					
	(A)combination 25/75		(B)combination 50/50		(C)combination 75/25		(A) 25/75		(B) 50/50		(C) 75/25		(A) 25/75		(B) 50/50		(C) 75/25	
	Alc. 250mg/ml	*Aqu. 500mg/ml*	*Alc.*	*Aqu.*	*Alc.*	*Aqu.*	*Alc.*	*Aqu*	*Alc.*	*Aqu.*	*Alc.*	*Aqu*	*Alc.*	*Aqu.*	*Alc.*	*Aqu.*	*Alc.*	*Aqu*
Erythromycin (0.01 mg/ml)	**17.1 aA**	**16.1 aA**	**13 bB**	**13.8 bA**	**10.8 cB**	**10.5 cA**	**18.2 abA**	**19.2 aA**	**16.1 cA**	**17.1 bcA**	**11 dB**	**11.2 dA**	**16.2 aA**	**14 bA**	**13 bcB**	**12 cA**	**10 dB**	**10.5 dB**
Gramicidin (0.01 mg/ml)	**14 abB**	**14 abB**	**15 aA**	**13.8 bA**	**12 cA**	**11.8 cA**	**18.2 aA**	**14 bB**	**17.3 aA**	**12.5 cB**	**15 bA**	**11.2 cA**	**13.5 bB**	**14 bA**	**16.5 aA**	**13 bcA**	**11.8 dA**	**12 cdA**

- According to Duncan's test, the statistical analysis shows that the same letters means that there are no significant differences among the results, while the different letters means the opposite.

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دراسة التأثير المضاد البكتيري لخلط بعض النباتات الطبية مع بعض المضادات على بكتريا ال *E. coli* المقاومة للمضادات المتعددة.

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المستخلص

بما أن الكائنات الدقيقة المقاومة سبب لمشكلة عالمية خطيرة، فقد بدأت النباتات الطبية بجذب انتباه العلماء لاستخدامها كأدوية بديلة أو كما في حال هذا البحث لخلطها مع المضادات الحيوية لإيجاد حل ضد أحد هذه الكائنات الدقيقة المقاومة. هذا البحث يبين إمكانية الحصول على نتائج إيجابية في تثبيط نمو بكتريا *E. coli* المقاومة للأدوية المتعددة عن طريق التآزر بين المستخلصات النباتات الطبية والمضادات الحيوية. تم خلط المستخلصات الكحولية والمائية لكل من نبات عرق السوس *Glycyrrhiza glabra* والدبق *Viscum album* والبابونج *Chamaemelum nobile* مع المضادات الحيوية: *Erythromycin ethylsuccinate* و Gramicidin كل منها على حدى وبنسب معينة للحصول على ثلاث أنواع من التوليفات (A و B و C). التوليفات التي احتوت على نسب أعلى من المستخلصات النباتية مقارنة بنسبة المضادات فيها أظهرت نتائج إيجابية تآزرية أعلى في تثبيط نمو بكتريا ال *E. coli* . علاوة على ذلك، بينت الكشوفات الكيميائية أن مستخلصات النباتات هذه تحتوي على مكونات فعالة معينة والتي بدورها تمتلك فعالية مضادة للميكروبات.

الكلمات المفتاحية: الدبق، عرق السوس، البابونج، التآزر، الفعالية المضادة للبكتريا، الأدوية البديلة، الأدوية الشعبية.