Synergistic Effect of Rosmarinus Officinalis Extract with Antibiotics against Different Bacterial Isolates

Thorria Raddam Marzoog
Biotechnology Division, Applied Science Department, University of Technology/Baghdad
Email:thmarzoog@yahoo.com

Received on: 13/12/2012 & Accepted on: 4/4/2013

ABSTRACT

Bacterial resistance to currently available antibiotics and its rapid increase is a worldwide concern. It is an even bigger problem since this resistance is not often restricted to a specific antibiotic, but generally extends to other compounds of the same class; therefore the need is urgent to develop new antimicrobial agents or new ways to treat the resistant microorganisms. Plants are inexhaustible source of natural antimicrobial compounds that have been found to be "synergistic enhancers" and this enables the use of the antibiotic when it is no longer effective by itself during therapeutic treatment.

The aim of this study was to determine the effect of combined application of Rosemary *(*Rosmarinus officinalis *L.*) ethanolic extract by its sub-inhibitory concentration with known antibiotics against different pathogenic isolates using disk diffusion method.

The results showed that the crude extract led to different effects (synergism, antagonism and indifference) on antimicrobial activity of the tested antibiotics. Synergistic effect was seen in most tested bacteria (63.33%), indifference effect was (26.67%) and antagonism effect was (10%) in tested isolates. These results signify that Rosemary extract potentiates the antimicrobial action of antibiotics, suggesting a possible utilization of this herb in combination therapy against pathogenic organisms.

التأثير التآزري لخلاصة اكليل الجبل Rosmarinus officinalis مع المضادات الحياتية ضد عزلات بكتيرية مختلفة

الخلاصة

يشكل ظهور سلالات بكتيرية مقاومة للمضادات الحياتية المستخدمة حالياً و زيادتها المتسارعة مصدر قلق في العالم اجمع ،ومما يزيد هذه المشكلة سوءاً كون هذه المقاومة لا تقتصر على مضاد حياتي معين بل تتعداه لتشمل كل المركبات التي تعود للصنف نفسه و لذا فالحاجة ملحة لأيجاد نوع جديد من المضادات الحياتية أو أيجاد طرق جديدة لعلاج الأحياء المجهرية المقاومة للمضادات .

تعتبر النباتات مصدر دائم للمركبات الطبيعية المضادة للأحياء المجهرية و التي تعتبر "معززة للتأثير التآزري"مما يجعل استخدام المضاد في العلاج ممكناً حتى عندما يفقد فعاليته العلاجية .

هدف هذا البحث هو دراسة تأثير الخلاصة الكحولية لنبات اكليل الجبل Rosmarinus officinalis عندما تمزج مع المضادات الحياتية المعروفة ضد عزلات مرضية بأستخدام طريقة الأنتشار بأقراص المضادات الحياتية .

أظهرت النتائج بأن استخدام الخلاصة النباتية ادت الى ظهور تأثيرات مختلفة (تـأثير تـآزري، مضـاد، غير مؤثر) علـى الفعاليـة الحياتيـة للمضـادات المسـتخدمة في الفحص، و كـان التـأثير الأكبـر هـو التـأثير التآزري 63.33% و غير المؤثر 26.67% ثم التـأثير المضـاد 10% . و قد دلـت هذه النتـائج علـى ان خلاصـة اكليل الجبل اعطت قوة تآزرية و حسنت من فعاليات المضـادات الحياتيـة ممـا يجعل اسـتخدام هذا العشب ممكناً بطريقة "العلاج المشترك"بأستخدام النباتات الطبية و المضادات الحياتية معاً لتحسين فعاليـة المضادات ضد السلالات المقاومة لها.

INTRODUCTION

The discovery of antibiotics had eradicate the infections that once ravaged humankind, but their indiscriminate use has led to the development of multidrug- resistant pathogens which present an ever increasing global health threat.

The pace of development of new antimicrobial agents has slowed down, while the prevalence of resistance has grown at an accelerating rate, and the past record of rapid, wide spread emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy, therefore, there is an urgent need to search for new classes of antimicrobial substances.

An alternative therapy to treat antibiotic- resistant microorganisms is "Combination Therapy" which uses a combination of plant extract and antibiotic against resistant pathogens.

This is a novel concept that has been recently ventured to overcome the resistance mechanisms of microbes [1, 2, 3].

Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with crude plant extracts against different pathogens [2, 4]. Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of therapeutic value [4]. According to the World Health Organization (WHO) in 2008, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs as drugs derived from natural sources play a significant role in the prevention and treatment of human diseases [5, 6].

The main advantage of natural agents is that they do not enhance the "antibiotic resistance" a phenomenon encountered with the long term use of synthetic antibiotics and they have been found to act as "synergistic enhancers" [1, 4, 6].The potential benefits of using combined antimicrobial therapy can be treatment of mixed infections, enhancement of antimicrobial activity, reducing the time needed for long-term antimicrobial therapy and prevention of the emergence of resistant microorganisms [5, 7, 8, 9].

Rosemary *(*Rosmarinus officinalis L.) is well known as a spice and a medicinal plant worldwide, it is a flowering plant belongs to family L*amiaceae* of herbs grows in Mediterranean countries, southern Europe and in the littoral region through Minor Asia areas wildly, and it is also known and grown in Iraq. Rosemary is confirmed medicinally for its powerful antibacterial antioxidant properties and as a chemopreventive agent [10, 11]).

The purpose of this study is to investigate an alternative treatment of bacterial infection by combining the antimicrobial properties of Rosmarinus officinalis L. extract

with standard antibiotics in order to obtain an improved antimicrobial effect against different strains of pathogenic bacteria.

Materials and Methods

Plant sample: Rosemary (Rosmarinus officinalis) leaves were collected from local garden, washed, dried in the shade at room temperature, ground using a coffee grinder and were used for extract preparation.

Preparation of plant extract: Soxholet extraction method [12]was used. Approximately 50 gm of dried plant material was extracted by adding 200ml of ethanol (96%) using soxholet apparatus of 500 ml for 7 hrs with continuous slow mixing. The extract solution was filtered and the solvent was removed using rotary evaporator at 45^{o}C to obtain the crude extract, and then it was kept in sterile bottles at 4^{o}C until use. The resulted deposit was dissolved in distilled water to prepare the doses.

Bacterial strains: The tested microorganisms were provided from the culture collection of Microbiology laboratory, Applied Science Faculty, University of Technology. These included: Staphylococcus aureus, Streptococcus faecalis, Bacillus subtilis, Escherichia coli, Pseudomonas aerugenosa, and Proteus mirabilis.

Antibiotics were selected with different targets on bacteria, Amoxicillin (25mcg), Ampicillin (10mcg), and Cephalothin (15mcg) as cell wall synthesis inhibitors, Tetracycline (30mcg) and Gentamicin (10mcg) as protein synthesis inhibitors.

Antimicrobial tests: Before the combination effect assay between plant extract and the antimicrobial drugs was evaluated, the antimicrobial activity of the extract was checked by determining the minimal inhibitory concentration (MIC) for the tested strains by diluting the extract in Mueller Hinton agar (MHA) media. Petri dishes with different concentrations of plant extract (mg/ml) and control, were inoculated with test strains (10^4 CFU) and were incubated at 37^{o}C/24 h. The concentration that inhibited visible growth of each strain (MIC) was recorded, and the MIC 90% was calculated.MIC_{90} is defined as the Minimum Inhibitory Concentration that required to inhibit the growth of 90% of organisms. One- fourth the MIC_{90} was considered as the sub- inhibitory concentration of the plant extract in the combination effect assay [13] which were carried out on test strains by disk diffusion method on MHA media.

A single colony of each test strain was grown overnight in nutrient broth tube and the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard (10^6 cfu/ml).Mueller Hinton agar (MHA) plates were spread with 0.1 ml of bacterial suspensions and left to dry at room temperature.

Standard antibiotic discs were applied to the inoculated MHA plates, in addition, another group of these antibiotic discs were saturated with MIC of extract per disc according to the value obtained previously for each bacterial strain, then placed on the inoculated (MHA) plates, and then they were incubated at 37^{o}C for 18 hr. At the end of the period, the diameters of inhibition zones were measured in mm. The assays were performed in triplicate.

Results and discussion

The Minimum Inhibitory Concentration MIC_{90} ($mg.ml^{-1}$) against test strains and one- fourth the MIC_{90} obtained before the combination effect assay for *Rosmarinus*

officinalis ethanol extract, are presented in table-1. The MIC_{90} range was 5-50 $mg.ml^{-1}$, and this difference is not surprising due to differences among bacterial species.

Sub- inhibitory concentration was used in the combined application of the extract and antibiotics to guarantee that the effect obtained was due to the combination not to the extract itself [12].

Table (1) MIC of Rosmarinus officinalis extract against test bacteria.

Bacteria	Inhibition Zones (mm)							
	Conc. Of *Rosmarinus officinalis* extract ($mg.ml^{-1}$)							
	100	50	25	10	5	1	MIC_{90}	Sub IC
Staph. aureus	–	–	–	±	+	++	10	2.5
Strep. feacalis	–	–	–	–	±	++	5	1.25
Bacillus subtilis	–	–	±	+	++	++	25	6.25
E. coli	–	–	–	±	++	++	10	2.25
P. auerogenosa	–	–	±	+	+	++	25	6.25
Proteus vulgaris	–	±	+	+	++	++	50	12.5

(–) :No growth, (±) :Minimum growth, (+) :Moderate growth, (++) :Maximum growth. Sub IC: Sub Inhibitory Concentration.

Rosmarinus officinalis ethanolic extract had antimicrobial effect on the majority of tested bacteria when combined with different antibiotics. Disc diffusion method was used to determine this effect as it serves as standard assay for measuring the activity of compounds against pathogenic bacteria.

The inhibition zones (diameter in mm) of antibiotics without *R. officinalis* extract against tested bacteria are shown in Table (2).

The inhibition zones (diameter in mm) of antibiotics in combination with *R.* officinalis extract against tested bacteria are shown in Table (3).

The combination of antibiotics with *R.* officinalis extract had different effects against tested bacteria (synergism, antagonism and indifference).Gentamicin with extract showed an increase in its antibacterial activity against all tested bacteria. Inhibition zone (mm) increased against: Staph. aureus from (24) to (28), Strep. feacalis (27) to (30), Bacillus subtilis (24) to (28), E. coli (18) to (20), P.aerugenosa (24) to (32) and Proteus vulgaris (10) to (12).Tetracycline with extract showed an increase in its antibacterial activity against: Staph. aureus from (21) to (24), Strep. feacalis (18) to (20), Bacillus subtilis (16) to (19), E. coli (14) to (18), but it showed no effect against P.aerugenosa and Proteus vulgaris .Ampicillin when combined with R. officinalis extract showed an increase in its antibacterial activity against: E. coli from (23) to (25) and P.aerugenosa (11) to (13), it showed a decrease in its antibacterial activity against: Staph. aureus from (28) to (23), Strep. feacalis (13) to (11) and there was no difference against Bacillus subtilis and Proteus vulgaris. There was an enhancement in Amoxicillin antibacterial activity when combined with extract, as it showed an increase in Inhibition zone against: Bacillus subtilis (10) to (13), P.aerugenosa (14) to (16) and Proteus vulgaris (18) to (21), it showed a decrease in inhibition zone against E. coli from (25) to (22) and no difference against Staph. aureus and Strep. feacalis. Finally,

Cephalothin which showed a synergetic reaction with extract against four of the tested bacteria as there was an increase in Inhibition zone against: Staph. aureus from (21) to (24), Strep. feacalis (21) to (23), Bacillus subtilis (15) to (17) and P.aerugenosa (18) to (21) while there was no difference against E. coli and Proteus vulgaris Table (2,3).

Table (4) shows the percentage of changes (increase or decrease) in antimicrobial activity due to combined applications.

The interaction between antibiotics and plant extract was mainly addition.

Table (2) Antibacterial activity of antibiotics on test bacteria.

Microorganism	Inhibition Zones (mm)				
	Cn	**Te**	**Am**	**Ax**	**C**
Staph. aureus	24	21	28	28	21
Strep. feacalis	27	18	13	18	21
Bacillus subtilis	24	16	13	10	15
E. coli	18	14	23	25	20
P. auerogenosa	24	R	11	14	18
Proteus vulgaris	10	R	R	18	19

Cn: Gentamicin, Te: Tetracycline, Am: Ampicillin, Ax: Amoxicillin, C: Cephalothin, R: Resistant

Table (3) Antibacterial activity of combination of Rosmarinus officinalis extract with antibiotics.

Microorganism	Inhibition Zones (mm)				
	Cn	**Te**	**Am**	**Ax**	**C**
Staph. aureus	28	24	23	28	24
Strep. feacalis	30	20	11	18	23
Bacillus subtilis	28	19	13	13	17
E. coli	20	18	25	22	20
P. auerogenosa	32	R	13	16	21
Proteus vulgaris	12	R	R	21	19

Cn: Gentamicin, Te: Tetracycline, Am: Ampicillin, Ax: Amoxicillin, C: Cephalothin, R: Resistant

Table (4) Percentage of synergistic/antagonistic effect of combination of Rosmarinus officinalis extract with antibiotics.

Microorganism	synergistic/antagonistic effect %				
	Cn	**Te**	**Am**	**Ax**	**C**
Staph. aureus	+16.66	+14.29	-17.85	ne	+14.29
Strep. feacalis	+11.11	+11.11	-15.38	ne	+9.52
Bacillus subtilis	+16.66	+18.75	ne	+30.0	+13.33
E. coli	+11.11	+28.57	+8.7	-12.0	ne
P. auerogenosa	+33.3	ne	+18.2	+14.3	+16.66
Proteus vulgaris	+20.0	ne	ne	+16.7	ne

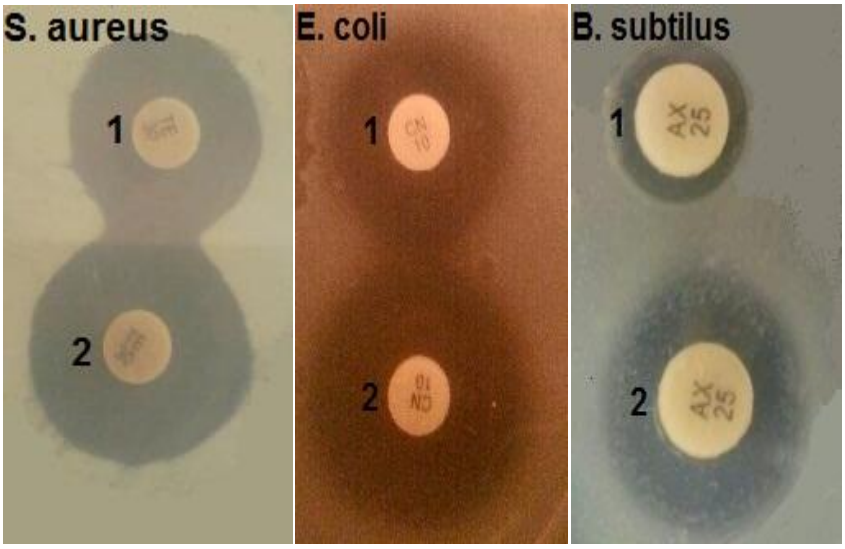
Cn: Gentamicin, Te: Tetracycline, Am: Ampicillin, Ax: Amoxicillin, C: Cephalothin
+: synergism, -: antagonism, ne: no effect.

Combination of extract with antibiotics showed synergistic effect on tested bacteria in most tests performed (63.33%) Table(4) Figure(1).

The increase in the inhibition zone was highest when the extract was combined with Gentamicin against Pseudomonas aerugenosa (33.3%), but it was the lowest against *Escherichia coli* with Ampicillin (8.7%).

Plant antimicrobials had been found that though they may not have a potent antimicrobial activity alone, but when they were taken concurrently with standard drugs they enhance the effect of that drug. [14, 15]

Antimicrobial mechanisms of the drugs used were variable; and the protein synthesis inhibitors (Gentamicin and tetracycline) were those that present strongest synergistic effect. Gentamicin showed synergism with extract against all test strains, followed by Tetracycline and Cephalothin. Amoxicillin showed an enhancement effect against three isolates with an increase as high as (30%)Figure (1) when combined with extract against *B. subtilus*, while Ampicillin showed synergism against only two of the isolates (E.coli and Pseudomonas aerugenosa)



Tetracycline Gentamicin Amoxicillin

Figure (1) Synergistic effect of R.officinalis extract and Antibiotics on test bacteria.

1: inhibition zone of the antibiotic alone.
2: inhibition zone of the antibiotic with R.officinalis extract
The reason of the enhancement effect is not known and needs investigation;

Previous studies referred that plant crud extracts have many different phytochemicals which might inhibit bacteria by different mechanisms. This double

attack of both agents on different target sites of bacteria could theoretically lead to either an additive or a synergistic effect. [14, 15, 16]

An antagonistic effect was seen in some of the tests performed (10%). The application of *R.* officinalis with Ampicillin led to decrease the antimicrobial activity against Staphylococcus aureus and Streptococcus faecalis, also the combination of the extract with Amoxicillin led to antagonistic effect against *Escherichia coli*, this effect may be due to numerous compounds within the crude extract that may have interfered with the action of one another, or it contained compounds that inhibited the antibacterial activity of the effective compounds,[13,16] so further separation and purification of the crude extract might show an increase in bioactivity than the crude extract.

On the other hand, there was no effect of the extract when combined with antibiotics against some test strains (26.67%).

It is thought that the observed differences may be a result of the combined effect of both active and inactive compounds of the extract, in addition, these differences in results among bacterial species may be due to the fact that the cell wall in Gram-positive bacteria is of a single layer, while the Gram- negative cell is a multi-layered structures [17, 18].

Interaction between known antibiotic and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction), or deleterious (antagonistic or toxic outcome) [18, 19, 20].

Previous reports mentioned a synergistic effect even though plant extracts did not show any activity by themselves, thus the researchers should investigate the synergistic capacity of plants extracts, independent of the antimicrobial activity they have, also different effects (synergism, antagonism and indifference) were mentioned between standard antibiotics and ethanolic extracts of different medicinal plants against pathogenic organisms. [21, 22, 23].

Drug- plant extract combinations had been shown to reduce and delay the emergency of bacterial resistance to antibiotics and may also produce desirable synergistic effects in the treatment of bacterial infections [23,24,25] generally the results obtained showed the potentials of *Rosmarinus officinalis* extract in the treatment of infectious diseases when combined with antibiotics and it further reduce drug resistance, also it revealed that the combination of the crude extract and the protein synthesis inhibitors had the highest inhibitory activity against bacterial isolates.

The evaluation of the exact drug- plant ratio at which the interaction is maximal between the plant extract and the antimicrobial drug, and the identification of the effective compounds in the crude extract, are necessary to examine the mechanisms of action of these agents especially that these mechanisms probably differ from those of the commonly used antibiotics.

REFERENCES

[1].Chanda, S. and Rakholiya, K:" Combination therapy: Synergism between natural plant extracts and antibiotics against infectious diseases". Science against microbial pathogens: communicating current research and technological advances. 520-529 (2011).

[2]. Toroglu S.: "In-vitro antimicrobial activity and synergistic/antagonistic effect of interactions between antibiotics and some spice essential oils", Journal of Environmental Biology, January 2011, 32 (1) 23-29 (2011).

[3]. Agnihotri, S. and Vaidya, A.D.: "A novel approach to study antibacterial properties of volatile components of selected Indian medicinal herbs".
Ind. J. Exp. Biol., 34, 712-715 (1996).

[4]. Alzoreky, N.S. and. Nakahara, K.:" Antibacterial activity of extracts from some edible plants commonly consumed in Asia". Int. J. Food Microbiol., 80, 223-230 (2003).

[5]. Toroglu, S., Digrak, M. and Kocabas, Y.Z.:" In vitro antimicrobial activity of essentialoils from Teucrium polium L., Thymbra spicata L. var. spicata, Ocimum basilicum L. and Foeniculum vulgare Miller consuming as tea or spice and their interactions withsome antibiotics". KSU. J. Sci. Engineer., 8, 36-42 (2005)

[6]. Kumral, A and Sahin, T.:" Effects of some spice extracts on Escherichia coli,
Salmonella typhimurium, Lisetiar monocytogenes, Yersinia enterocolitica and
Enterobacter aerogenes". Ann. Microbiol, 53, 427-435 (2003).

[7]. Dulger, B. and Gonuz, A.:" Antimicrobial activity of certain plants used in Turkishtraditional medicine". Asian J. Plants Sci., 3, 104-107 (2004).

[8]. Erdogrul O.T.: "Antibacterial activities of some plant extracts used in Folk medicine".Pharm. Biol... 40, 269-273 (2002).

[9]. Toroglu, S., Digrak, M. and Cenet, M.:" Determination of antimicrobial activities of essential oils of consumed for spice Laurus nobilis Linn and Zingiber officinaleRoscoe and theirs effects on antibiotics in vitro". KSU. J. Sci. Engineer., 9, 20-26 (2006)

[10]. Faixove, Z, Faix, S.:" Biological effects of Rosmarinus officinalis L. essential oil".Folia Veterinaria.52, 3-4: 153-139,(2008).

[11]. Derwich,E., Benziane,Z. and Chabir,R.:" Aromatic and medicinal plants of Morocco: Chemical composition of essential oils of Rosmarinus officinalis and Juniperus phoenicea".Inter.J. App.Bio.Pharm.Tech., 2, 1, 145- 153 (2011).

[12]. Genena, A., Hense,H., Junior,A.,Souza,M.,:" Rosemary (Rosmarinus officinalis L.) – a study of the composition, antioxidant and antimicrobial activities of extracts obtained with supercritical carbon dioxide".Cienc.Technol. Aliment.28, 2, (2008).De, M., A.K. De, R.

[13]. Betoni,J, Mantovani,R., Barbosa,L., Stasi,L.,Junior,A.,:"Synergism between plant extract and antimicrobial drugs used on Staphylococcus aureus diseases". Mem.Inst.Oswaldo Cruz. 101,4,(2006).

[14]. Bonjar, G.H.S.: "Evaluation of antibacterial properties of Iranian medicinal plantsagainst Micrococcus luteus, Serratia marcescens, Klebsiella pneumoniae and
Bordetella bronchoseptica". Asian J. Plant Sci., 3, 82-86 (2004b).

.[15]. Nascimento, G. F., Locatelli, J., Freitas, P.C. and Silva, G.L.: "Antibacterial
activity of plant extracts and phytochemicals on antibiotic-resistant bacteria".
Braz.J. Microbiol. Oct. /Dec. Vol. 31, No.4. ISSN 1517-8382 (2000).

[16]. Wei Wang, Nan Li, Meng Luo, Yuangang Zu and Thomas Efferth: "Antibacterial Activity and Anticancer Activity of Rosmarinus officinalis L. Essential Oil Compared toThat of Its Main Components". Molecules, 17, 2704-2713, (2012).

[17]. Toroglu,S.:"In-vitro antimicrobial activity and synergistic/antagonistic effect of interactions between antibiotics and some spice essential oils". Journal of Environmental BiologyJanuary 2011, 32 (1) 23-29 (2011).

[18].Stojanovic- Radc,Z., Nesic,M., Comic,L.and Radulovic,N.: "Antimicrobial activity and cytotoxicity of commercial rosemary essential oil (Rosmarinus officinalis L.)".10th SFSES. 17-20 June 2010, Viasina lake.

[19].. Oluwatuyi, M.; Kaatz, G.W.,Gibbons, S. :"Antibacterial and resistance modifying activity of Rosmarinus officinalis". Phytochemistry, 65, 3249–3254. 2004.

[20].Abascal, K. and Yarnell, E.: Herbs and drug resistance. Potential of botanical in Drug-resistant microbes. Alternative Complementary Therapies, 1, 237-241 (2002).

[21]. Mothana, R.A.A., Gruenert, P. Bernarski, J. and Lindequist U.:" Evaluation ofthe in vitro anticancer, antimicrobial and antioxidant activities of some Yemeni plants used in folk medicine". Pharmazie, 64, 260-268 (2009).

[22]. Coates A, Hu YM, Bax R, Page C. "The future challenges facing the development of new antimicrobial drugs". Nature Reviews Drug Discovery. 1:895-910. 2002.

[23]. Moghaddam KM, Iranshahi M, Yazdi MC, Shahverdi AR. "The combination effect of curcumin with different antibiotics against Staphylococcus aureus". Int J Green Pharm; 3:141-3. 2009.

[24]. Adwan,G., Mhanna, M.: "Synergistic effect of plant extracts and antibiotics on Staphylococcus aureus strains isolated from clinical specimens". Middle –East J. Scientific Research 3 (3):134- 139. (2008).

[25]. Mukhopadhyay, A. Banerjee, B. and Miro, Y.M.:"Antimicrobial activity of Cuminum cyminum L". Ars. Pharmaceutica, 44, 257-269 (2003).