The Activity of Extracts of *Rosmarinus officinals* and *Eucalyptus spathulata* Hook Leaves on the Growth of *Escherichia coli* and *Candida albicans* with Estimation of the Median Lethal Dose of Both Extracts in Laboratory Mice

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Abstract

This study was conducted to investigate the activity of alcoholic extract of leaves of rosemary, *Rosmarinus officinalis* and *Eucalyptus spathulata* on the in vitro growth inhibition of *Escherichia coli* and *Candida albicans* by agar well diffusion method, with estimation of median lethal dose of both extracts. Alcoholic extract of both leaves with three concentrations (100, 200 and 300 mg/ml) were prepared. The three concentrations of both plants extracts showed high effect on the in vitro inhibition of growth of *E. coli.* The means of inhibition zone diameters were 13.2, 15.2 and 18.4 mm for *R. officinalis* and 17.2, 18.2 and 20 mm for *E. spathulata*. There was moderate effect of the three concentrations of both extracts on the growth of *C. albicans*, the means of inhibition zone diameters were10.2, 12.3 and 14.6 mm for *R. officinalis* and 13.4, 15.2 and 16 mm for *E. spathulata*. The results of estimation of median lethal dose (LD_{50}) indicated that there was no lethal effect of both leaves extracts in the laboratory mice, with the exception of the appearance of loss of appetite, increase respiratory and heart rates and ruffled hair without mortality at the doses ranged between 9000-20000 mg/kg body weight.

الخلاصة

أجريت هذه الدراسة لكشف فعالية المستخلصات الكحولية لأوراق نباتي إكليل الجبل *Rosmarinus officinalis* والكالبتوس *Eucalyptus spathulata* في تثبيط نمو جرثومة الاشيريشا القالونية وخميرة المبيضات البيض خارج الجسم بطريقة الانتشار بالحفر مع تقدير الجرعة القاتلة الوسطية لكلا المستخلصين. حضرت ثلاثة تراكيز من المستخلص الكحولي لأوراق النباتين (300,200,100 ملغم/ مل) .أظهرت التراكيز الثلاثة لكلا المستخلصين تأثيرا عاليا في تثبيط نمو جرثومة الاشيريشيا القولونية وبمعدلات أقطار تثبيط قدرها 18.4,15.2,13.2 ملم لتراكيز مستخلص أوراق إكليل الجبل و 20,18.2,17.2 ملم لتراكيز مستخلص أوراق الكالبتوس على التوالي، كما أشارت النتائج إلى وجود تأثير متوسط الفعالية للتراكيز الثلاثة لكل المستخلصين في تثبيط نمو خميرة المبيضات البيض حيث بلغت معدلات أقطار التثبيط 14.6,12.3,10.2 ملم لكل تركيز من تراكيز مستخلص أوراق إكليل الجبل و16,15.2,13.4 ملم لكل تركيز من تراكيز مستخلص أوراق اليوكالبتوس على التوالي. أظهرت نتائج تقدير الجرعة الوسطية القاتلة لمستخلصي أوراق النباتين عدم وجود تأثير قاتل لكلا المستخلصين في الفئران المختبرية عدا ظهور أعراض سريريه تمثلت بفقدان الشهية وزيادة معدل التنفس و ضربات القلب مع شعر مجعد و عدم ظهور أية حالات موت في حدود الجرعات التي تراوحت بين 9000الى 20000 ملغم/كغم من وزن الجسم.

Introduction

In the last ten years, there has been considerable increase in mycoses and systemic infections caused by *C. albicans* particularly among immunocompromised patients (Eggimann, *et al.*, 2003). Studies have shown an increased resistance of *C. albicans* to azoles such as fluconazole (Rex, *et al*., 1995) and to Amphotercin B. (Kaughman and Carver, 1997).

Plant extracts as well as essential oils are of considerable interest because of their antifungal activity (Lima, *et al.*, 1993) and plant-derived poly phenols receive high attention because of their potential antimicrobial properties (Proestos, *et al.*, 2006).

Rosemary (*Rosmerinus officinalis L.*) is a spice and medicinal herb widely used around the world. Rosemary essential oil is also used as an antibacterial and antifungal (Oluwatuyi, *et al.*, 2004). The main compounds responsible for the antimicrobial activity are α-pinene, camphor and 1,8-cineole (Daferera, *et al.*, 2000).

E. spathulata leaves are lanceolate apparently alternate and waxy or glossy green (Brooker, M. and Kleinig, D., 2006). The essential oils extracted from eucalyptus have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant activities (Burt, 2004) and also have been used in cancer treatment (Sylvestre, 2006).

The present study describes the antimicrobial activity of leaves extracts of rosemary and eucalyptus on the *E. coli* and *C. albicans*, with estimation of LD_{50} for both extracts in laboratory mice.

Materials and Methods

Preparation of Plant Leaves

Rosemary and eucalyptus leaves were obtained and dried. They were ground separately to a fine powder in a mixer for ten seconds, the particle size distribution was determined with a vibratory sieve shaker. The ground particles were stored under vacuum and maintained in freezer -20 C° until use.

Extraction by Ethanol 95%

Eighteen gm of dry plant powder was placed in a beaker and 150 ml of ethanol 95% was added to it and left on magnetic stirrer for two hours. The whole mixture was filtered first by medical gauze followed by centrifugation at 3000 rpm for 15 seconds. Supernatant was collected and put in carthen bowl to dryness by oven at 40 C°. The crude extract was kept at -20 C° until use (Fehri, *et al.*, 1994).

Preparation of Alcoholic Extract Concentrations

One, two and three gm was weighed from the dry primary crude extract and each weight was dissolved in 10 ml of ethanol 95%. Three concentrations were prepared as 100, 200 and 300 mg/ml. Each concentration was put on vortex for 10 seconds, sterilized by millipore filter (0.22 µm) and then put in sterile tubes for use.

The Antimicrobial Activity of Plant Leaves Extracts

Agar well diffusion was used to determine the activity of each plant extract in vitro against *E. coli* and *C. albicans*. Four pure colonies were suspended into four ml of nutrient broth and incubated for 2-8 hours at 37 C°. The turbidity of inoculation was standardized with Mac Farland tube No. 1 containing 1.5 x 10^8 cfu/ml. The suspended cells were swabbed on nutrient agar. Five wells were made in agar using a sterile cork borer (6 mm) with micropipette. Two hundred micro liters of each plant concentration were poured in three wells (three plates for each concentration) and ethylene glycol was poured in the fourth well as a control. The plates were incubated at 37 C° for 24 hours. Diameters of inhibition zone were measured (Mahmood, *et al.*, 1989). For antifungal activity, two colonies of *C. albicans* were suspended in saline solution. The turbidity was adjusted to equal 0.5 Mac Farland standards. (1x 10^5 to 1x10^6 cfu/ml). The suspended cells were swabbed on Meuller-Hinton agar supplemented with 2% glucose and methylene blue (0.5 µg/ml). Five wells were made and 200 µml of each concentration of plant extract was poured in the fourth well (three plates for each concentration) and

ethylene glycol was poured in the fifth well as a control. The all plates were incubated at 35 C° for 48 hours. The diameters of inhibition zones were recorded in milliliter (Griggs, *et al.*, 2001).

Estimation of Median Lethal Dose (LD_{50}) of Plant Extracts

According to the procedure described by Dixon, (1980) up-down method was used to determine the LD_{50} of alcoholic extract of both plant leaves. Different plant extracts concentrations ranged from 1000-20000 mg/ml body weight with various numbers of white mice were used. This test calls for dosing individual animals in sequence singly at 24 hours intervals, with the initial dose set at the toxicologist best estimate of LD_{50}. Following each death or moribund state, the dose was lowered and following each survival, it was increased according to pre specified dose progression factor. If death follows an initial direction of increasing doses of 25%, a survival follows an initial direction of decreasing doses with the same ratio.

Results

The results showed that the three concentrations of extracts plays important role in the inhibition zone diameters. The increasing of these diameters is related to the increase in extracts concentrations. The three concentrations of rosemary leaves extracts gave the mean inhibition zone diameters of 13.2, 15.2 and 18.4 mm against *E. coli*, while the same concentration of eucalyptus extracts gave 17.2, 18.4 and 20 mm, respectively against the same bacteria. (Tables 1 and figures 1 and 2).

The three concentrations of alcoholic extract of rosemary leaves against *C. albicans* showed that the inhibition zone diameters were 10.2, 12.3 and 15.2 mm , while the inhibition zone diameters of alcoholic extracts of eucalyptus leaves against the same yeast were 13.4, 15.2 and 16 mm, respectively (Tables 2 and figures 3 and 4).

According to up-down method for determination of LD_{50} of alcoholic extract of rosemary and eucalyptus leaves with doses ranged from (1000-20000) mg/kg body weight, the results showed that there is no lethal effect due to these extracts on the laboratory mice, with the exception of appearance of some clinical signs such as loss of appetite, increase respiratory and heart rates, ruffled hair without mortality in doses ranged between 9000-20000 mg/kg body weight in both extracts.

Table (1): The in vitro activity of alcoholic extract of rosemary and eucalyptus leaves against *E. coli*.

Plant extracts	Concentration of extract (mg/ml)	Mean inhibition zone of diameters ± SE
Rosemary leaves	100	13.2 ± 0.3
	200	15.2 ± 0.3
	300	18.4 ± 0.5
Eucalyptus	100	17.2 ± 0.2
	200	18.4 ± 0.2
	300	20.0 ± 0.2

Table (2): The in vitro activity of alcoholic extract of rosemary and eucalyptus leaves against *C. albicans*.

Plant extracts	Concentrations of extract (mg/ml)	Mean inhibition zone of diameters ± SE
Rosemary leaves	100	10.2 ± 0.2
	200	12.3 ± 0.4
	300	15.2 ± 0.5
Eucalyptus leaves	100	13.4 ± 0.3
	200	15.2 ± 0.5
	300	16.0 ± 0.2

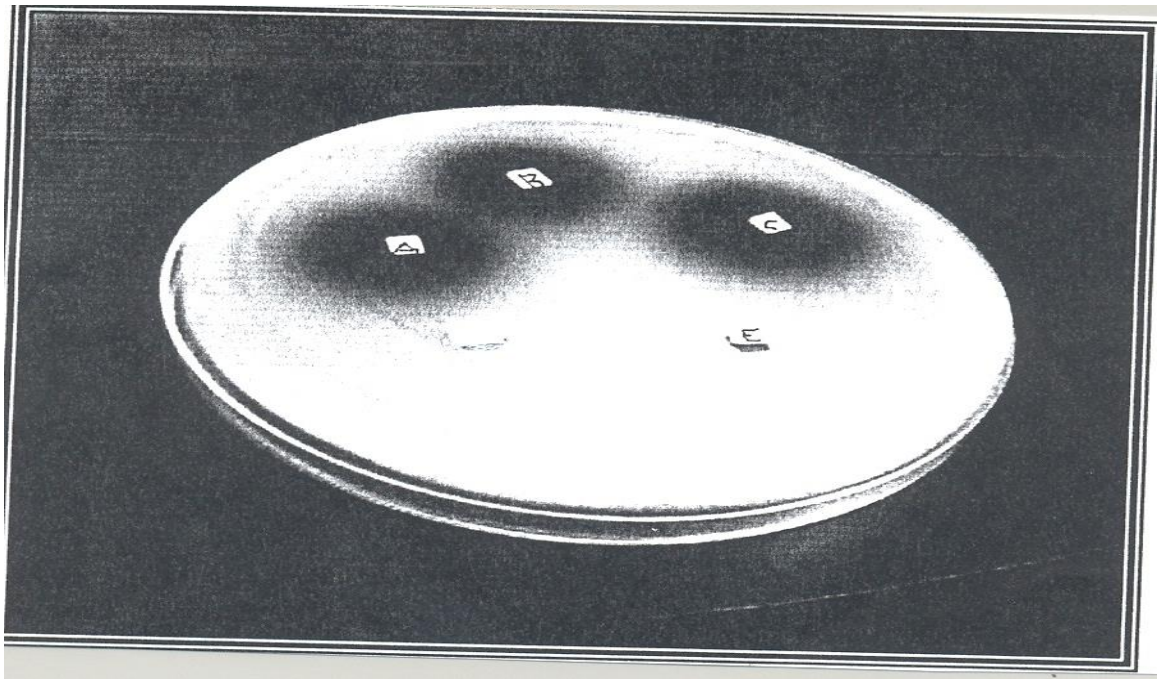

Figure (1): The effects of three concentrations of rosemary leaves extracts in the growth of *E. coli*.

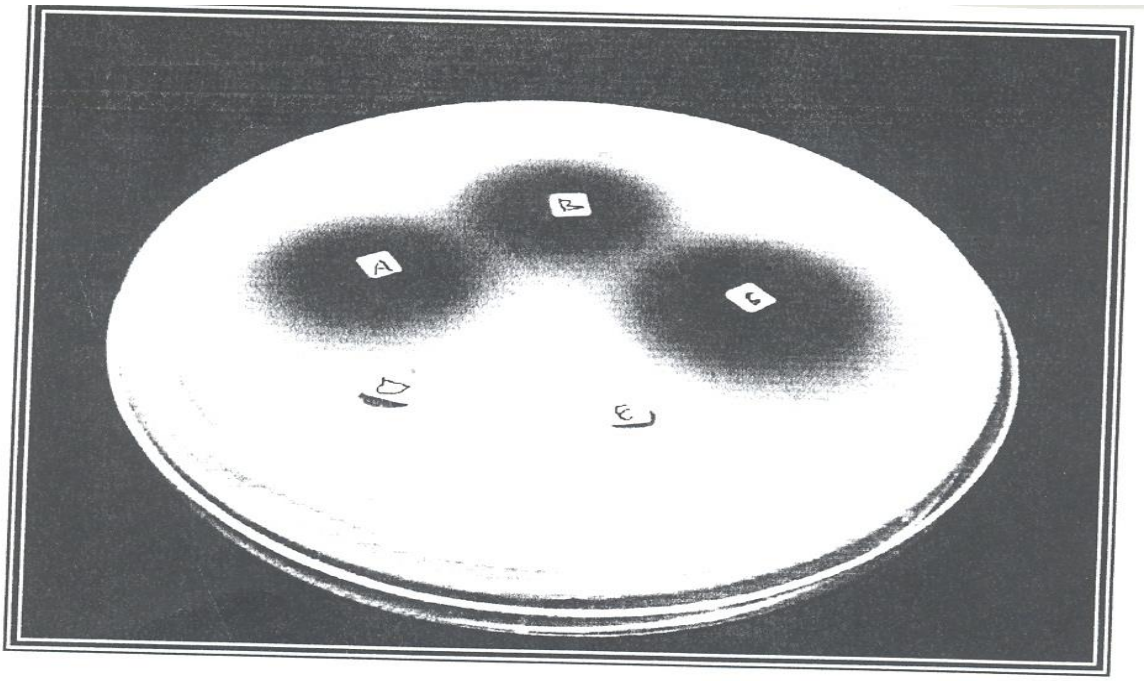

Figure (2): The effects of three concentrations of eucalyptus leaves extracts in the growth of *E. coli*.

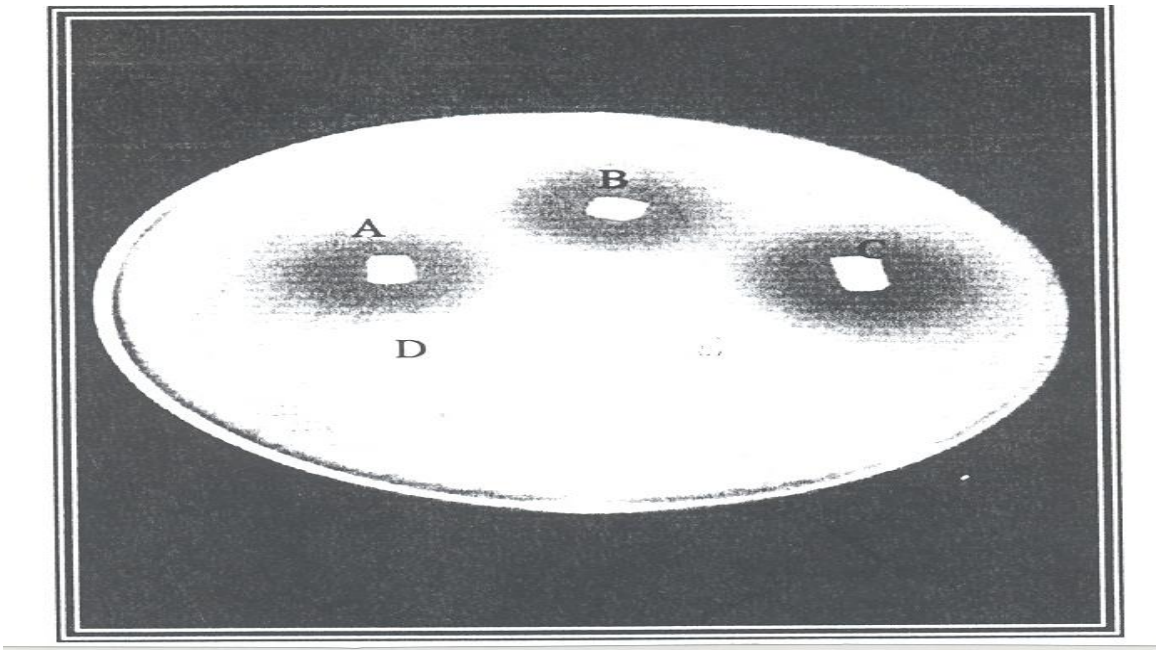

Figure (3): The effects of three concentrations of rosemary leaves extracts in the growth of *C. albicans*.

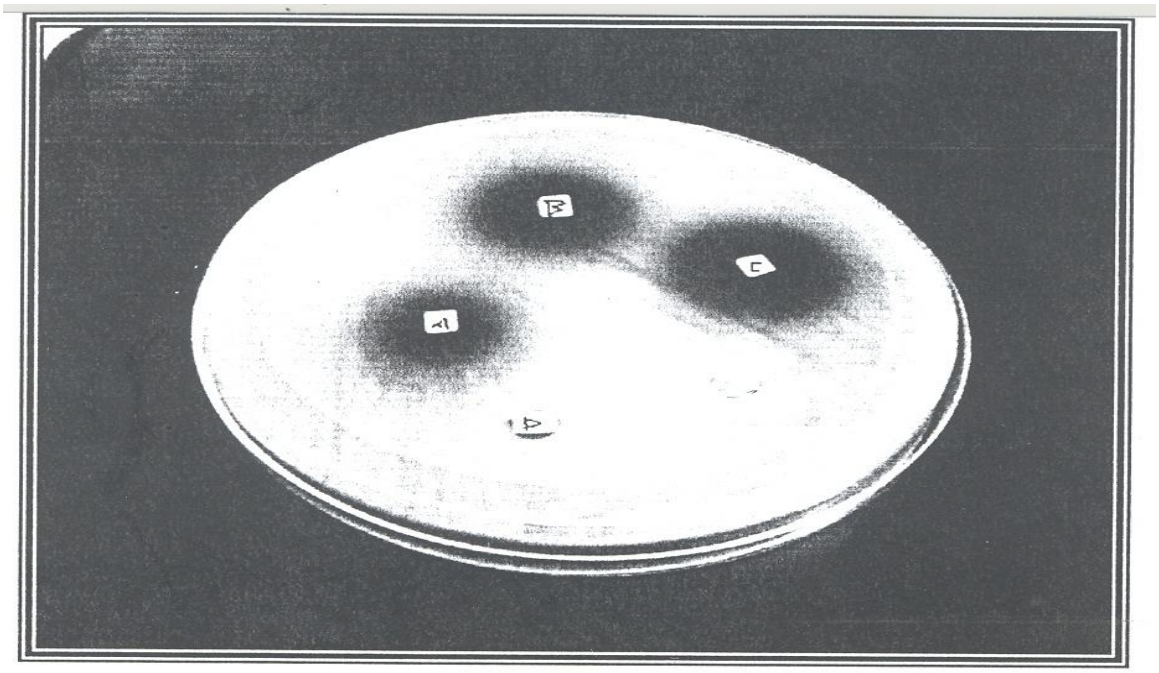

Figure (4): The effects of three concentrations of eucalyptus leaves extracts in the growth of *C. albicans*.

Discussion

The alcoholic extraction of both plant leaves showed antibacterial activity against *E. coli* growth and antifungal activity against *C. albicans* (Table 1 and table 2).

A good to moderate antimicrobial activity of rosemary has been reported by several authors (Celiktas, 2007), (Quispe, 2005) and (Gachkar, 2007). As the extract of rosemary leaves contains α-pinene and camphor, which are responsible for antimicrobial activity (Daferera, *et al.*, 2003). Here the activity by which substances phenol or resins and alkaloids (Tyler *et al.*, 1988), have active effect against bacteria by its ability to form hydrogenous bond with protein which lead to stopping protein built up in the cell (Burt, 2004). These results came in agreement with what was mentioned by (زنكنه، 2004)and (مصطفى، 1996).

The results of the present study suggest that rosemary extract had moderate effect on the growth of *C. albicans*. The main components of rosemary are 1,8-cineol and α-pinene. The antimicrobial activities of α-pinene are due to a change of membrane permeability arising from membrane alteration (Bouzouita, 2005). The percentage of 1-8 eucalyptus affects the anticandidal effect of the oil mixture negatively because rosemary oil had eucalyptol and α-pinene affect on membrane permeability, but the anticandidal activity of rosemary has decreased. Larrondo and Calvo,(1991) showed that rosemary oil has no effect on *C. albicans*. The phenolic compound of eucalyptus could be considered, clinically as antifungal agent. The emergence of multiple resistant *C. albicans* is important to find an effective treatment for infection due to these organisms.

The results showed that the leaves extracts of both plants caused no any mortality in laboratory mice. There are many factors, which affect the estimation of LD_{50} of plant extracts, such as the differences in sources of the plants, differences in chemical components, type and strain of laboratory mice, the method of dose calculation and other circumstances of experimentation. So, both plant extracts of the present study can be considered as safe treatment by oral administration (Lomis, 1968).

In vivo studies are necessary to assess potential therapeutic effects of both plant extracts.

References

Bouzouita N, Kachouri F, Hamdi, M. and Chaabouni M, (2005.)Volatile constituents and microbial activity of *lavandula stoechas L.* oil from Tunisia. J. Essential Oil Research

Brooker M and Kleinig D, (2006.) Field Guide to Eucalyptus Melbourne: Bloomings. South Eastern Australia. Third edition.

Burt, S, (2004) Essential oils: their antibacterial properties and potential applications in foods- a review. International Journal of food microbiology, Utrecht, 49(3): 223-253.

Celiktas O, (2007). Antimicrobial activities of methanol extracts and essential oils of *Rosemarinus officinalis*, depending on location and seasonal variations. Food chemistry, Bornova-Izmir/ Eskisehir 100 (2): 553-559.

Daferera D, Ziogas B and Polissiou M, (2000). GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *pencillium digitatum*. Journal of Agricultural and food chemistry, Athens, 48 (6): 2576-2581.

Daferera D, Ziogas B and Polissiou M, (2003). The effectiveness of plant essential oils in the growth of *Botrytis cinerea*, Fusarium sp. and Clavibucter michiganensis subsp. Crop production, Athens, 22 (1):39-44.

Dixon WJ, Efficient analysis of experimental observation. Ann. Rev pharmacol. Toxical. 1980; 20: 441-462.

Egyimann P, Carbino J and Pittet D, (2003). Epidemiology of *candida albicans* species infections in critically ill non-immunosuppressed patients. Lancet Infect. Dis.: 685-702.

Fehri B, Aiache J, Memmi A, Korbi S and Lamaison J, (1994). Hypotension, Hypoglycemia, hypouricemia recorded after repeated administration of aqueous leaf extract of oleaeuropael. J. Pharm. Belg. 49 (2): 101-109.

Gachker L, (2007).Chemical and biological characteristics of *Cuminium cyminum* and *Rosmarinus officinalis* essential oils. Food chemistry, Tehran.; 120:898-904.

Griggs J, Manandhar N and Tayler R, (2001). The effects of storage on the biological activity of medicinal plants from Nepal. J. Ethnopharmacol. 77: 247-252.

Kaughman C and Carver P, (1997). Antifungal agents in the 1990s. Current status and Future Developments. Drugs, 53: 539-549.

Larrondo J and Calvo M, (1991). Effect of essential oils on *C. albicans*: A scanning electron microscope study. Biomed. Letters. 46: 269-272.

Lima E, Gompertz O, Giesbrecht A And Paulo M, (1993). In vitro antifungal of essential oil obtained from officinal plants against dermatophytes. Mycosis. 36: 333-336.

Lomis A, (1968). Essentials of toxicology. Ph.D. M.D. University of Washington, School of Medicine Seattle, Washington, Philadelphia.

Mahmood MJ, Jawed AJ, Hassain AM, Al-Omeri, M and Al-Naib A, (1989). In vitro antimicrobial activity of *Salsola rosmarinus* and *Adiantum capillus venris*. Int. J. Crude Drug Res. 27: 14-16.

Oluwatuyi M, Kaatz WG and Gibbons S, (2004). Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry. London/ Detroit, 95 (24): 3249-3254.

Proestos C, Seroli D and Komaitis M , (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. Food chemistry Athens, 95 (1): 44-54.

Quispe-Condori S, (2005). Global yield isotherms and kinetic of artemisinin extraction from *Artemisia annua L*. leaves using supercritical carbon dioxide. Journal of supercritical fluids, Sao Paulo/ Hamburg, 36 (1): 44-52.

Rex JH, Rinald MG and Pfaller MA, (1995). Resistance of *candida* species to fluconazole. Antimicrob. Agents Chemother. 39: 1-8.

Sylvestre M, Pichette A, Longtin A, Nagau F and Legault J, (2006). Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens L*. from Guadeloupe. J. Ethnopharmacol. 103: 99-102.

Tayler VE, Brady LR and Robbers JE, (1988). Pharmacognosy, 9th ed., Philadelphia, Lea and Febiger, Goowashington square, Philadelphia, USA.

زكنة، شكرية علي محمود.(2004). " تأثير مستخلصات عدد من النباتات على نمو بعض أنواع البكتريا المرضية". رسالة ماجستير/كلية العلوم– جامعة الانبار.

مصطفى، أيمان عبد العزيز.(1995). " التأثيرات البايولوجية المثبطة لمستخلصات بعض النباتات الطبية في بعض الأحياء الدقيقة المعزولة من قنوات جذور الأسنان". رسالة ماجستير/كلية العلوم– جامعة الموصل.