Curing of mice skin infections using ethanol flower extract of chamomile

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Abstract:

This experiment was conducted in order to estimate azulene and apigenin in chamomile flowers. Ethanol extracts were examined singly or in combination with some drugs in their biological activity against some pathogens causing skin infection. Ethanol extract was applied at a concentration of 40 mg/ml for the treatment of induced skin infection of mice. Among the topicals used, Claforan was found the most effective on microorganisms causing skin diseases; ethanol extract was more effective than the drug Candimazole solution 1%. HPLC was used for the determination of azulene and apigenin active compounds of chamomile plant.

Key words: chamomile flowers; antimicrobial activity, skin infections

Introduction:

German Chamomile (*Matricaria recutita*) is a daisy-like flower that blooms from late spring through late summer; it is an annual plant of the composite family Asteraceae [1]. An infusion of the flowers is taken internally as an anti-inflammatory, antiseptic, antispasmodic, carminative, diaphoretic, febrifuge, sedative, stomachic, tonic and vasodilator [2, 3, 4]. The flowers are also used externally to treat wounds, sunburn, burns, haemorrhoids, mastitis and leg ulcers. An infusion is particularly useful as a stomachic, nervine and sedative for young children, especially when they are teething [4].

Skin infections are common and may be caused by bacteria, fungi or viruses. Breaks in the skin integrity, particularly those that inoculate pathogens into the dermis, frequently cause or exacerbate skin infections [5]. The *in vivo* study of possible therapeutic effect of chamomile extract on bacterial infections was performed on mice skin. The chamomile extracts were prepared and applied locally on the skin of experimentally infected mice described by [6].

Drugs used for skin infections are Claforan (cefotaxime sodium) which has an *in vitro* activity against a wide range of Gram-positive and Gram-negative microorganisms [7], Acetic acid 6%, also known as ethanoic acid, is an organic chemical compound, giving sour taste and pungent smell [8]. Fusidic acid is another drug only effective on Gram-positive bacteria such as *Staphylococcus spp*. and *Corynebacterium spp*., it inhibits bacterial replication and does not kill the bacteria and is therefore termed "bacteriostatic" [9]. Clotrimazole (Candimazole) solution 1% was also used for treatment of susceptible fungal infections including oropharyngeal candidiasis, dermatophytoses, superficial mycoses and cutaneous candidiasis [10].

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HPLC is used for the determination of flavonoids and has become a popular method for separation, screening and quantitative analysis of plant, food products and also herbal medicines [11, 12, 13]. This paper addresses the potential of using chamomile flower ethanol extract in treatment of skin inflammations compared with some commonly used drugs.

Materials and Methods:

Chamomile dried flowers were bought from a local market and identified as a German chamomile by specialist. The microorganisms *Candida albicans*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from Biotechnology Dept., College of Science, Al-Nahrain University.

A quantity of 50g of flowers powder was extracted with 250 ml of 75% ethanol by soxhlet apparatus for 6 hrs. at 40-60ºC. The suspension was filtered through a filter paper (Whatman no.1), and then the solvent was removed under reduced pressure by using rotary evaporator at 40ºC, filter-sterilized using Millipore unit (0.22µm). The crude solid extract (24g) was kept in a deep freeze until use [14].

The activity of the extracts was determined against target microorganisms (*S. aureus*, *P. aeruginosa* and *C. albicans*) *in vitro* by using modified agar diffusion method describe by [15].

The extract was prepared at three concentrations 10, 20 or 40 mg/ml. The dried alcohol extract was redissolved with alcohol (75% ethanol).

The Tryptone soya agar medium was mixed well and aliquots of 20 ml were poured in Petri-dishes. The medium was inoculated with 0.1 ml of (1.5×10^8 CFU/ml) target isolates of *S. aureus*, *P. aeruginosa* or *C. albicans* by using sterile swabs.

Four evenly spaced wells, 3 mm in diameter were made in the agar of each plate with sterile cork borer. To identify the intrinsic extracts activity, one control well was filled with (100 µl) phosphate buffer saline (PBS). Equal volumes of the three concentrations 10, 20 or 40 mg/ml of the extracts were dispensed into each well (three replica plates were prepared for each agent). Test plates were then incubated at 37°C for 24 hrs. and zones of inhibition were measured using a ruler. A clear zone indicated that the extract showed its antibacterial or antifungal activity. This method was repeated three times for each test. (The values were averaged for the three experiments).

The activity of the drugs (Cefotaxime sodium, Fusidic acid, Clotrimazole and Acetic acid 6%) that were used for treatment of human skin infection was investigated. The effect of chamomile flower ethanol extract individually or combined with these drugs were determined by using modified agar diffusion method.

In this study, two groups (A and B) of mice were experimented; each group included twelve mice which were divided into four subgroups represented by three mice for each. The experiment was conducted as follows: The hair on the back of mice was shaved, and the area was cleaned and disinfected with cotton swab saturated with 70% alcohol, direct scraping of the skin was done by sterile pathological scalpel to make abrasion on one half of the mouse's back skin and the skin of each group was subjected to the infections as stated below (mice were treated with different treatments daily):

Group A: represented by twelve mice, this was divided into four subgroups representing three mice for each. The

scratched skin infected with 0.1 ml of *S. aureus* suspension ($1.5x10^8$ CFU/ml by McFarland) from an overnight *S. aureus* grown culture. The signs of a wound infection exhibited redness, swelling, and pus appeared after 10 days.

The first subgroup: each of the three mice was smeared by a suspension of *S. aureus* and after ten days of the infection, the infected skin was treated with 0.1 ml (the drug Cefotaxime sodium 125mg/ml + chamomile flower ethanol extract 20mg/ml) at 50% from each. This group was used for infection and treatment of mice.

The second subgroup: each of the three mice was smeared by a suspension of *S. aureus* and after ten days of the infection, the infected skin was treated with 0.1 ml of the drug Cefotaxime sodium (250mg/ml).

The third subgroup: each of the three mice was smeared by a suspension of *S. aureus* and after ten days of the infection, the infected skin was treated with 0.1 ml of the chamomile flower ethanol extract (40mg/ml).

The fourth subgroup: each of the three mice was smeared by a suspension of *S. aureus* and after ten days of the infection, the infected skin was treated with 0.1 ml of PBS.

Group B: represented by twelve mice, this was divided into four subgroups representing three mice for each. The scratched skin infected with 0.1 ml of *C. albicans* suspension ($1.5x10^8$ CFU/ml) from *C. albicans* culture. The signs of a wound infection appeared as redness, swelling, and pus after 10 days.

The first subgroup: each of the three mice was smeared by a suspension of *C. albicans* and after ten days of the infection, the infected skin was treated with 0.1 ml (Clotrimazole 0.75mg/ml + chamomile flower ethanol extract 10mg/ml) at 75% from each. This group was used for infection then treatment of mice.

The Second subgroup: each of the three mice was smeared by a suspension of *C. albicans* and after ten days of the infection, the infected skin was treated with 0.1 ml of 1% Clotrimazole.

The third subgroup: each of the three mice was smeared by a suspension of *C. albicans* and after ten days of the infection, the infected skin was treated with 0.1 ml of the chamomile flower ethanol extract (40mg/ml).

The fourth subgroup: each of the three mice was smeared by a suspension of *C. albicans* and after ten days of the infection, the infected skin was treated with 0.1 ml of PBS.

HPLC analysis

Azulene in ethanol extracts was estimated by gradient HPLC. Chromatography conditions: column Tessek SGX C18 7 μm (4 ¥ 250 mm); flow rate 0.7 ml·min1; mobile phase A: acetonitrile/water/H_3PO_4 (19:80:1), B: 45% acetonitrile, C: 90% acetonitrile. The linear gradient elution programme from 100% A to 100% B for 25 min, then to 100% C for 30 min, isocratic for 5 min, and returning to 100% A for 45 min. Detection was performed at 320 nm of wavelength as described by [16].

Apigenin standard was dissolved in methanol. A stock solution of 0.567 mg/ml was prepared. For HPLC calibration curve, concentrations ranged from 0.0567 to 0.567 mg/ml were prepared.

The analytical method was used with some modifications. Samples were separated on a reversed phase column, Symmetry C18 column (3.9´ 150 mm; 5 mm particle size), manufactured by Waters, USA. The mobile phase consisted of methanol and water in a volume ratio of 50:50 with a flow rate of 0.8 ml/min.; all samples were passed through a 0.45μm Millipore filter as described by [17].

Results:

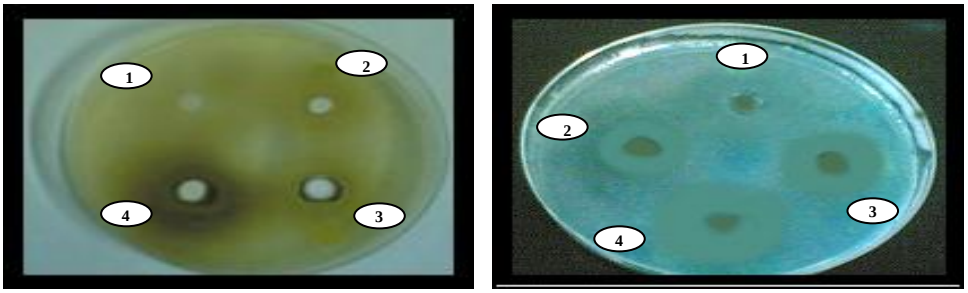
Chamomile flowers ethanol extract exhibited antibacterial activity against microorganisms at the concentration 10mg/ml (table 1) and (Fig. 1). The diameter of the inhibition zones against *S. aureus* was 12.1 mm at concentration of 10 mg/ml. The higher concentrations of extracts 20 and 40 mg/ml, *S. aureus* showed 13.2 mm and 15.0 mm, respectively. Results also showed that *P. aeruginosa* gave 29.7 mm inhibition zones at 10 mg/ml. While the concentrations of extracts showed 33.2 mm at 20 mg/ml and 37.4 mm at 40 mg/ml of the chamomile flowers ethanol extract.

In *Candida*, the low concentration 10 mg/ml showed 21.1 mm inhibition zones subsequently, 28.1 and 30.4 mm were recorded at the concentrations of 20 and 40 mg/ml.

Table 1: Diameter of inhibition zones caused by chamomile flower ethanol extract at various concentrations on *S. aureus*, *P. aeruginosa* and *C. albicans*

Concentration mg dwt/ml	Diameter of inhibition zone (mm) ± S.D.		
	S. aureus	*P. aeruginosa*	*C. albicans*
Control (PBS)	-ve	-ve	-ve
10	12.1±0.14	29.7±0.25	21.1±0.07
20	13.2±0.12	33.2±0.68	28.1±0.46
40	15.0±0.10	37.4±0.10	30.4±0.07

-ve: no activity was observed
Values are mean of 3 replicates ± S.D.



-a- -b-

Fig. 1: Inhibition zones (mm) caused by different concentrations of chamomile flower ethanol extract -a- *S. aureus*, -b- *P. aeruginosa*. 1= control (PBS), 2= 10, 3=20, 4= 40.

Table (2) showed the *in vitro* effect of chamomile ethanol extract on the antibacterial activity of some drugs and treatments that used for curing skin of wounds. Cefotaxime sodium:ethanol extract 50% recorded the highest diameter of inhibition zone (70.0 mm) against *S. aureus*, but Cefotaxime sodium:ethanol extract 75% (187.5:10)mg/ml recorded an inhibition zone reached 61.0 mm against *P. aeruginosa* while Cefotaxime sodium:ethanol extract at 25% (62.5:30)mg/ml achieved the highest diameter of inhibition zone (32.1) mm against *C. albicans*.

Acetic acid at 100% achieved the highest diameter of inhibition zone (22.0 mm) against *S. aureus*, but Acetic acid at 25% (1.5:30)mg/ml gave an inhibition zone 13.2 mm against *P. aeruginosa* but the ethanol extract was better than Acetic acid in a diameter of inhibition zone (29.7 mm). The Acetic acid 75% (4.5:10)mg/ml achieved the diameter of inhibition zone (29.0 mm) while ethanol extract alone produced the largest inhibition zone (33.5 mm) against *C. albicans*.

Fusidic acid at 100% achieved the highest diameter of inhibition zone (40.5) against *S. aureus*, but no inhibition zone against *P. aeruginosa*, while giving an inhibition zone 30.5 mm against C. albicans.

Clotrimazole was tested against C. albicans only. At percentage 75% (0.75:10)mg/ml achieved a diameter of inhibition zone reached 24.3 mm compared with the ethanol extract

which recorded 33.5 mm inhibition zone and with the Clotrimazole at 100% which recorded 28.4 mm. The ethanol extract of chamomile at a concentration of 40 mg/ml and drugs (Cefotaxime sodium and Clotrimazole) showed an obvious destroying effect on *S. aureus* and *C. albicans* in experimentally induced skin infection in mice compared to controls (infection). The infection showed swallowing, redness and filled with pus cells as shown in table (3), (4) and Fig. (2) which represents 2 groups of mice, group A was infected with *S. aureus*, and group B was infected with *C. albicans*.

The results showed that the better treatment is (Cefotaxime sodium and chamomile ethanol extracts) at 50%, as in table (2) and the results showed that the better treatment is (Chamomile flower ethanol extract) more than Clotrimazole at as in table (3).

Table 2: Antimicrobial activity expressed as a diameter of inhibition zone caused by chamomile flower ethanol extract in combination with some antimicrobial drugs i*n vitro*.

Drugs (%)	Final concentrations (mg/ml)	Diameter of inhibition zone (mm) ± S.D.		
		S. aureus	*P. aeruginosa*	*C. albicans*
Cefotaxime sodium:extract				
25:75	62.5:30	60.1±1.05	48.0±1.05	32.1±1.00
50:50	125:20	70.0±1.00	50.2±2.00	31.0±1.00
75:25	187.5:10	65.1±0.95	61.0±1.00	28.3±0.20
100:0	Extract only 250:0	46.0±1.00	48.1±1.00	36.2±0.95
0:100	alcohol extract 40	30.1±1.00	29.7±1.00	33.5±2.00
Acetic acid 6%:extract				
25:75	1.5:30	18.1±1.00	13.2±0.86	22.5±1.00
50:50	3:20	19.0±1.00	15.5±1.00	27.1±1.00
75:25	4.5:10	20.1±1.10	19.0±1.00	29.0±2.00
100:0	Extract only 6%:0	22.0±1.00	24.0±1.00	20.0±1.00
0:100	alcohol extract 40	30.1±1.00	29.7±1.00	33.5±2.00
Fusidic acid:extract				
25:75	5000:30	36.0±1.00	-	32.1±1.00
50:50	10000:20	39.1±1.00	-	40.2±1.10
75:25	15000:10	37.2±1.00	-	37.2±1.00
100:0	Extract only 20000:0	40.5±1.00	-	30.5±1.00
0:100	alcohol extract 40	30.1±1.00	29.7±1.00	33.5±2.00
Clotrimazole:extract				
25:75	0.25:30	*	*	24.0±1.00
50:50	0.50:20	*	*	21.1±1.00
75:25	0.75:10	*	*	24.3±1.00
100:0	Extract only 1%:0	*	*	28.4±1.00
0:100	alcohol extract 40	*	*	33.5±2.00

Values are mean of 3 replicates ± S.D.
*** Not tested since Clotrimazole is a fungicide- No inhibition zone**

Table 3: Effect of different treatments on curing of mice skin infected with *S. aureus* in four experimental mice subgroups

Sub grouping of experimental mice (group A)	Types o treatment	Result
First subgroup	(Cefotaxime sodium and chamomile flower ethanol extract) at 50%	Complete cure after 16 days
Second subgroup	Cefotaxime sodium	Complete cure after 17 days
Third subgroup	Chamomile flower ethanol extract	Complete cure after 22 days
Fourth subgroup	PBS	Died after 12 days

Table 4: Effect of different treatments on curing of mice skin infected with *C. albicans* in four experimental mice subgroups

Sub grouping of experimental mice (group B)	Type of treatment	Result
First subgroup	(Clotrimazole and chamomile flower ethanol extract) at 75%	Complete cure after 20 days
Second subgroup	Clotrimazole	Complete cure after 25 days
Third subgroup	Chamomile flower ethanol extract	Complete cure after 10 days
Fourth subgroup	PBS	Died after 8 days

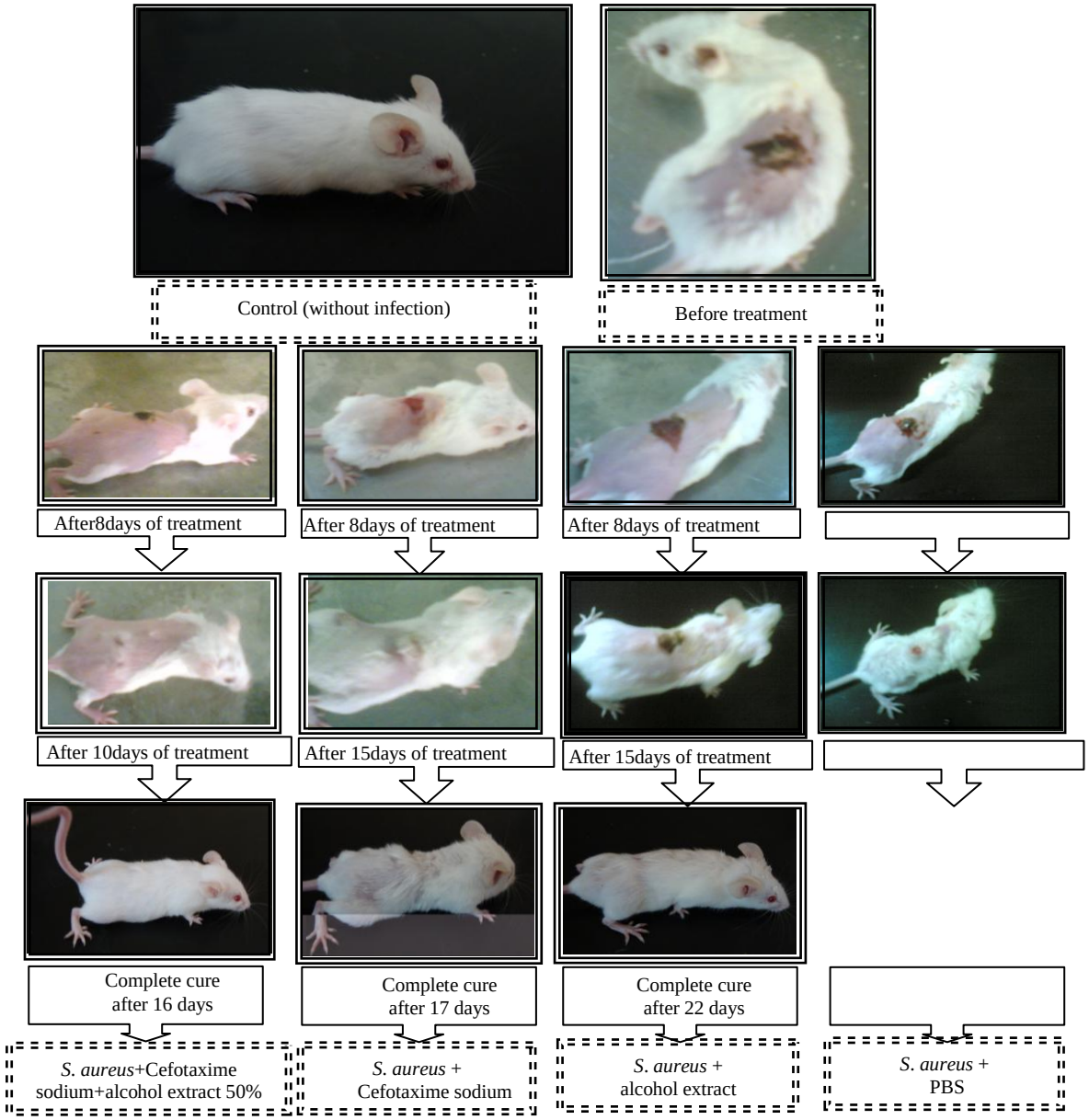


Fig. 2: Morphological repair of the skin infected by *S. aureus* and treated with a combination of Cefotaxime sodium + chamomile flower alcohol extract at 50%, Cefotaxime sodium, chamomile flower alcohol extract and PBS.

HPLC chromatography of the used standards (apigenin and azulene) is shown in Fig. (3). Apigenin appeared at a retention time of 7.60 min. with a peak area of 1973157 mAU, while azulene appeared at a retention time of 20.72 min. with a peak area of 778417 mAU.

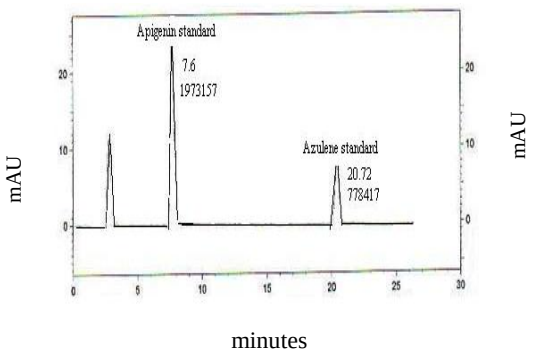


Fig. 3: HPLC analysis of apigenin and azulene standards

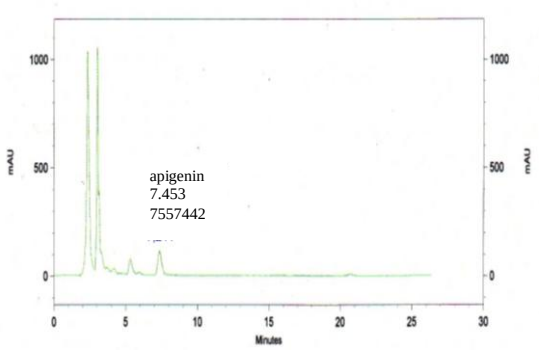


Fig. 4: HPLC of apigenin and azulene in chamomile flower ethanol extract showing appearance of the first compound and disappearance of the second.

Chamomile alcohol extract of apigenin and azulene is shown in Fig (4). Apigenin appeared at a retention time of 7.453 min. with a peak area 7557442 mAU while azulene was not detected at a retention time of 7.453 min. The percentage of apigenin in alcohol extract represented 0.3064% of the crude extract.

Discussion:

Results displayed in table (1) were in agreement with AL-naymi [18] who reported that ethanol extracts of chamomile flowers have higher activity than water ones, suggesting that the activity of chamomile could be attributed to the existence of chamazulene, α- bisabolol (sesquiterpenes) that showed high inhibition activity against *S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Micrococcuse ssp.* and *C. albicans.*

Results exhibited in table 2 indicated that Cefotaxime sodium has the highest inhibition on *S. aureus* and *P. aeruginosa*, but the most effective treatment against *C. albicans* was Clotrimazole solution, because the Cefotaxime sodium has high activity against microorganisms and the Clotrimazole solution has high activity against *C. albicans.* This may be due to the absorption of Cefotaxime sodium and Clotrimazole solution by skin (*in vivo*) during the treatment, and its activity had increased after addition of chamomile extract.

Results showed in table (3) and Fig. (2) exhibited that skin treated with Cefotaxime sodium used a complete cure after 16 days which was better than those treated with chamomile flower ethanol extracts.

Table (4) showed that mice skin treated with chamomile ethanol extracts was better since it rapidly recovered than those treated with the Clotrimazole. It appears that the presence of apigenin, the most active compound of chamomile, penetrates into deeper skin layers when applied topically which supports the use of chamomile as a topical anti-inflammatory agent in treating inflammations in deep tissues [19, 20].

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معالجة جلد الفئران المصابة ببعض المسببات البكتيرية والفطرية بالمستخلص الايثانولي لأزهار البابونج

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الخلاصة:

نفذت تجربة لتقدير الازولين والابجنين في أزهار البابونج. اختبرت الفعالية البيولوجية للمستخلصات الايثانولية لازهار نبات البابونج *Chamomilla recutita* بشكل منفرد او توليفه مع بعض العقاقير لدراسة فعالياتها الحيوية ضد بعض المسببات المرضية لامراض الجلد المستحثة في الفئران البيض. أثبتت النتائج أمكانية استعمال المستخلص الايثانولي للبابونج عند التركيز 40 ملغم/مليليتر لمعالجة اصابات الجلد المستحثة. كما استخدم العقار الكلوفيران وكان الاكثر فعالية ضد أصابات الجلد البكتيرية، اضافة الى محلول الكاندي مازول لمعالجة اصابات الجلد الفطرية. أثبت المستخلص الايثانولي فعالية في معالجة الاصابات الجلدية للفئران البيض مقارنة مع محلول الكاندي مازول تركيز1%. وقد استعملت تقنية HPLC لتقدير مادتي الابجنين والازولين في المستخلص الايثانولي.