Antibacterial Activity of Chloroform Extract from Tagetes Erecta L. Flowers

Zainab Aziz Ali, Thamer M. Jasim*, Widad M K Alani**
College of pharmacy, Mustansiriyah University, Baghdad, Iraq
****Ashur University College, Department of pharmacy, Baghdad, Iraq***

DOI: https://doi.org/10.32947/ajps.19.04.0414

Article Info:

Received 5 May 2019
Accepted 4 Aug 2019
Published 1 Nov 2019

Corresponding Author email:
Pharm.dr.thamer@uomustansiriyah.edu.iq
orcid: https://orcid.org/0000-0002-6070-4718

Abstract:

Antibacterial activity of chloroform of T. erecta flower extract was performed on six-gram negative bacteria species (Serratia marcescens, Acinobacter Spp., Escherichia coli, Salmonella Spp., Morganella morganii, Pseudomonas aeruginosa) and two-gram positive Bacteria (Streptococcus pneumonia and Staphylococcus aureus). The antibacterial study was performed by ager well diffusion and disk diffusion methods. The extract shows good results against examined bacteria and the results obtained by agar well diffusion method was better than those obtained from disk diffusion method.

Key words: chloroform extract, agar well diffusion method, disk agar diffusion method.

الفعالية البكتيرية لمستخلص الكلوروفورم لزهرة الجعفري

زينب عزيز علي، ثامر مطلك جاسم*، وداد العاني**
**** كلية الصيدلة، الجامعة المستنصرية، بغداد، العراق***
***** كلية اشور الجامعة، قسم الصيدلة، بغداد، العراق***

الخلاصة:

تم فحص الفعالية البكتيرية لمستخلص الكلوروفروم لزهرة نبات الجعفري على ستة انواع من البكتيريا السالبة لصبغة غرام (Serratia marcescens, Acinobacter Spp., Escherichia coli, Salmonella Spp., Morganella morganii, Pseudomonas aeruginosa) ونوعين من البكتيريا الموجبة لصبغة غرام (Streptococcus pneumonia and Staphylococcus aureus). من خلال طريقة الاكار وطريقة الدسك واظهر المستخلص فعالية بكتيرية جيده ضد جميع انواع البكتيريا المستخدمه بالدراسة وكانت نتائج طريقة الاكار افضل من نتائج طريقة الدسك.

الكلمات المفتاحية: مستخلص الكلوروفورم، طريقة الاكار، طريقة الدسك.

Introduction

T. erecta L.is one of the commercially exploited flower crops of the genus *Tagetes* and family *Asteraceae. T. erecta* L. is popularly known as African marigold. *T. erecta* is native to Mexico and naturalized in rest of Central America and south America, also it is naturalized in the tropics and is widely cultivated all over the world like Africa and Indian Ocean islands, also it is cultivated widely in Iraq [1]. Biochemical studies show that *T. erecta* leafs and flowers are rich in alkaloids, flavonoids, tannins and essential oils [2]. These active ingredients have very important antibacterial activity. A plant of medicinal use since prehistoric times. In Mexico, decoction of flowers and leaves used as diuretic and carminative [3]. This plant is widely used as antibacterial [4], larvecidal [5], insecticidal [6], antifungal [7] and Nematicidal [8]. Despite of availability

of antibacterial agents now a day, but there uses are limited by number of factors like side effects, drug toxicity, and the most important factor is the emergence of resistant strains of bacteria. This phenomenon result in many clinical problems including the disease treatment caused by these microorganisms. Therefore, it is important to discover new, safer, and more effective antibacterial agents from natural sources like plants. The aim of this study is to evaluate the antibacterial activity of *T. erecta* flower chloroform extract against bacteria.

Materials and Methods

Collection of plant:

Flowers of *T. erecta* plant were collected from AL-Itifia nursery during March and April of 2018.

Preparation of extracts:

The flowers of *T. erecta* were dried under shade for about ten days. The dried flowers were pulverized by electric mill, 100 gram of powdered plant flowers extracted with 1500 mL of chloroform after defatting with hexane using soxhlet apparatus. The extracts filtered and evaporated to dryness under reduced pressure using rotary evaporator. The extract filtered and evaporated to dryness under reduced pressure using rotary evaporator and were left at 4 ^{0}C until assessment for their antimicrobial activities. The stock solution of chloroform (2 gram/ 2 ml) was prepared by dissolving 2 grams of chloroform extract in 2 ml of dimethyl-sulfoxide (DMSO). The dilution serials of 1 gram/ml, 500 mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml were prepared for antibacterial assay by agar well diffusion method. Stock solution of chloroform 1 gram/ 1ml was prepared by dissolving 1 gram of chloroform extract in 1 ml of dimethyl-sulfoxide. The dilution serials of 500 mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml, 31.25mg/ml were prepared for antibacterial assay by disk diffusion method.

Tested microorganisms:

Six-gram negative bacteria species (*Serratia marcescens, Acinobacter Spp., Escherichia coli, Salmonella Spp., Morganella morganii, Pseudomonas aeruginosa*) and two-gram positive bacteria (*Streptococcus pneumonia* and *Staphylococcus aureus*) were tested. The isolates were obtained from educational laboratory /College of pharmacy / Mustansiriyah University. They were isolated from different clinical sources.

Antibiotic sensitivity test:

The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections [9]. The antibiotics susceptibility test of the tested organisms was performed by disk diffusion method [10,11]. The antibiotics used were: *Trimethoprim* (TMP-10mcg), *Cefixime* (CFM-5mcg), *Nitrofurantoin* (F-100mcg), *Doxycycline* (DO-10mcg) ,*Amoxicillin/Clavulanic Acid* (AMC- 20/10 mcg), *Rifampin* (RA- 5mcg) and *Ofloxacin* (OFX-5mcg).

Agar well diffusion bioassay:

The antibacterial activity of chloroform extract was determined by agar well diffusion method and carried out by using pure culture for all species of bacteria. Stock cultures of the test bacteria were grown in Muller Hinton Broth (MHB, Merck, Germany) medium at 37˚C for 22 hours. The final cell concentrations were adjusted to 1.5 × 10^{8} CFU/ML with reference to the McFarland turbid meter [12,13]. The media were allowed to solidify, and wells were prepared in the seeded agar plates with the help of a cup borer (6 mm) into agar. Subsequently, in each agar plate of tested bacteria eight wells were made and (100µl) of dilutions of the extracts (1 gram/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml) introduced into wells on the plate. The DMSO was used as the negative control, and *Cefixime* was used as positive control.

The plates were kept at 37 °C for 24 hours and the antimicrobial action was estimated by determining the diameter of the inhibition zone. Evaluation of antibacterial action was based on extent of the diameter of inhibition zone formed all over the place of the well.

Disk diffusion bioassay:

The antibacterial activity of chloroform extract was determined by disk diffusion method. Discs of 6 mm in diameter were punched out using Whatman No. 1 filter paper with the aid of a paper punch and placed in Bijou bottles. The discs were sterilized by autoclaving at 121 ^{0}C for 15 min after which they were allowed to cool. Stock solution of the chloroform extract was prepared by dissolving 1 g in 1 ml of dimethyl sulphoxide. Therefore, stock solution had a concentration of 1000mg/ml. From this stock, five different concentrations of the extract were prepared, these are 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml which finally yielded disc potencies of 500 mg/disk, 250 mg/disk, 125 mg/disk, 62.5 mg/disk and 31.25 mg/disk respectively. This was followed by introducing 100 sterile discs into each concentration. The discs were allowed to absorb the solution and sept for further analysis. Each paper disc was capable of absorbing 0.01 ml. The bacterial plates prepared by the same method as in well diffusion agar method using the same type and number of bacteria but without making wells in the plate. Subsequently, in each plate of tested bacteria disks of dilutions of the extracts (500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml) introduced into the plate. The DMSO was used as the negative controller. The plates were kept at 37 °C for 24 hours and the antimicrobial action was estimated by determining the diameter of the inhibition zone. Evaluation of antibacterial action was based on extent of the diameter of inhibition zone formed all over the place of the disk.

Results and discussion:

Antibiotic sensitivity test:

It seems that all types of bacteria are resistant to rifampin while completely sensitive to *Ofloxacin* except *Streptococcus pneumonia* which show resistance to all antibiotics used in the tests. Other involved antibiotics varied in their responses towards all types of bacteria tested and these results considered as positive control in this study, as shown in Table (1) and Figure (1).

Table(1): Antibiotic sensitivity test.

Bacteria species	Antibiotic code/ concentration in microgram(mcg)/ inhibition zone in millimeters						
	AMC (20/10)	DO (10)	F (100)	CFM (5)	TMP (10)	RF (5)	OFX (5)
Serratia marcescens	0	6	7	0	0	0	20
Acinobacter Spp	5	7	0	0	5	0	9
Escherichia coli	0	4	4	5	20	0	13
Salmonella Spp	0	6	6	0	25	0	10
Morganella morganii	0	0	3	0	0	2	6
Pseudomonas aeruginosa	8	0	0	20	20	2	20
Streptococcus pneumonia	0	0	0	0	2	0	3
Staphylococcus aureus	5	0	0	9	12	2	14

Amoxicillin/Clavulanic Acid (AMC- 20/10 mcg), Doxycycline (DO- 10mcg), Rifampin (RA- 5mcg), Cefixime (CFM-5mcg), Trimethoprim (TMP-10mcg), Nitrofurantoin (RF-100mcg), and Ofloxacin (OFX-5mcg).

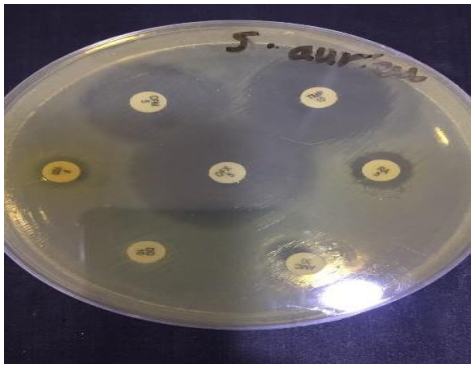

Figure (1): Antibiotic sensitivity test results

Anti-bacterial assay of chloroform extract of *T. erecta* flower by agar well diffusion method: The chloroform extract of *T. erecta* flower were screened for their antibacterial activity by agar well diffusion method and DMSO used as a negative control against six gram negative bacteria (*Serratia marcescens, A. Spp., E.coli, S. Spp, M. morganii, P. aeruginosa*) and two gram positive bacteria (*S. pneumonia* and *S. aureus*) at concentration of 1000mg /ml, 500 mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml of chloroform extract as shown in Figure (2) and Table (2).

The chloroform extract shows variable results at different concentration towards bacteria. The weakest concentration was 1000 mg/ ml; therefore, this concentration was not used in the rest of the study. while the most effective concentration was the lowest one 31.25mg/ml followed by 62.5mg/ml and the rests concentrations show moderate activity against all bacteria understudy. The most resistant bacteria to chloroform extract was *E. coli* and the most sensitive one was *S. marcescens*. This study show that chloroform extract has good antibacterial study and this result is confirmed by other studies in the world, one of these studies show that chloroform has an excellent antibacterial activity against *s. aureus* [14] while another study show that chloroform extract has good antibacterial activity against *E. coli* and *P. aeruginosa* [15]. The essential oil and terpenes content of *T. erecta* chloroform extract may be responsible for the antibacterial activity. The presence of terpenes and volatile oil in *T. erecta* flowers confirmed by gas chromatography as shown in Fig (3). The terpenes, Stigma-Sterol at retention time 26.602 and Oleic acid at retention time 18.079are detected by gas chromatography in chloroform extract. The structures of Stigma-Sterol and Oleic acid are shown in Figures (4) and (5) and all their detailed GC information are illustrated in Tables (3) and (4). They are responsible for the antibacterial activity and this activity was confirmed by other studies in the word, one of these studies show that stigma sterol has antibacterial activity by using acetone extract of the roots of *Salvia jaminiana*, containing the sterols: stigma sterol and Sitosterol remarkably inhibited the growth of Bacillus subtilis and S. aureus [16]. While other study confirmed the antibacterial activity of oleic acid by using dichloromethane extract of leaves of *Helichrysum pedunculatum* resulted in the isolation of oleic acids. Oleic acid was active against Gram-positive bacteria (*S. aureus* and *Micrococcus kristinae)* at a MIC of 1.0 mg/ml while it was inactive against the Gram-negative species tested [17]. The essential oil antibacterial activity was confirmed by other studies one of these studies show that the essential oil of *T. erecta* exhibited moderate antimicrobial activity against *Bacillus subtilis* and *Bacillus anthraces* while slight activity was observed against *Escherichia coli, Klebsiella pneumoniae, Salmonella pullorum, Salmonella richmond, Salmonella newport, Salmonella stanley, Salmonella typhimolium, Staphylococcus aureus, Proteus vulgaris, Pseudomonas agalactiae.* [18]. Thus, this study is confirmed by other studies in the world

and show the antibacterial activity of *T. erecta* flower active ingredients (stigma sterol, oleic acid and essential oil).

Figure (2): Results of Antibacterial activity of *T. erecta* chloroform extract by agar well diffusion method.

Table 2: Antibacterial activity of *T. erecta* chloroform extract with different bacterial species measured in millimeter by agar well diffusion method.

Bacterial species	Concentrations of chloroform extracts (mg/ml)/inhibition zone in millimeters						Positive Control	Negative Control
	1000	500	250	125	62.5	31.25	Cefixime	DMSO
Serratia marcescens	5	7	7	5	5	5	0	0.5
Acinobacter Spp	0	5	5	5	10	5	0	0.5
Escherichia coli	0	0	5	0	1	1	0.1	0
Salmonella Spp	0	10	0	0	15	10	1	0.5
Morganella morganii	2	3	5	5	0	5	0	0.5
Pseudomonas aeruginosa	0	0	0	5	5	7.5	0	0.3
Streptococcus pneumonia	3	3	5	7	2	6	0	0
Staphylococcus aureus	0	0	0	1	0.75	1	1.5	0

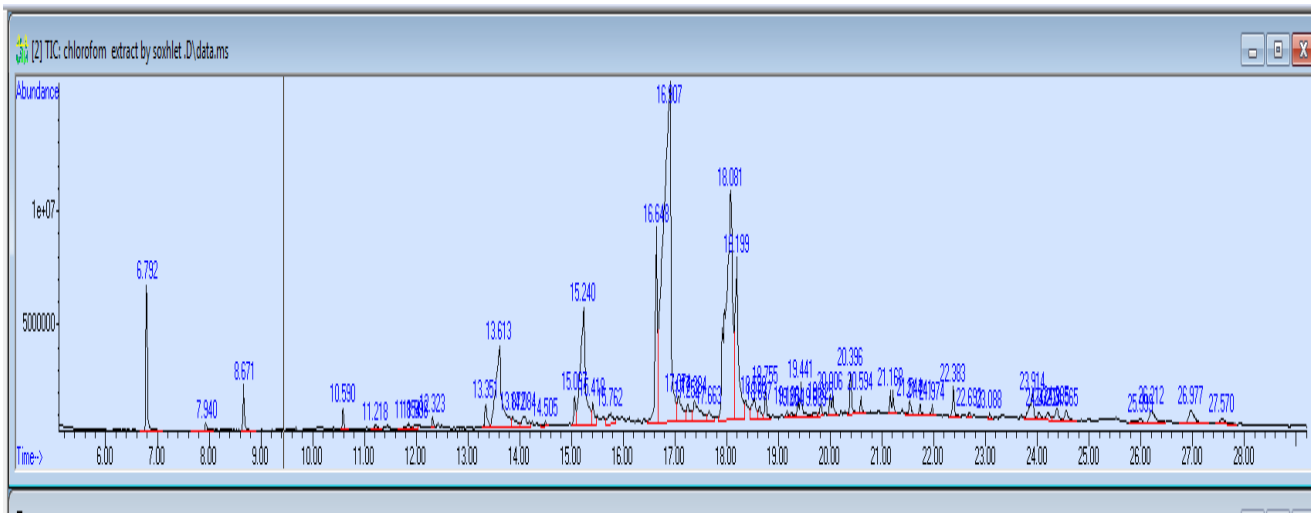

Figure (3): GC chromatogram of chloroform extract of *T. erecta* by Soxhlet

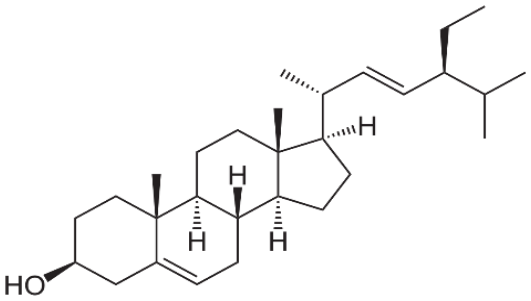

Fig (4): Chemical structure of Stigma-Sterol

Table (3): GC information of Stigma- Sterol

Retention time	26.602
Similarity index	92
Molecular weight	412.702 g/mole
Molecular formula	$C_{29}H_{48}O$
Library ID	Stigmasterin
Cass number	83-48-7

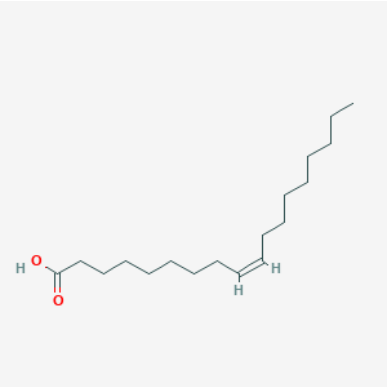

Fig (5): Chemical structure of Oleic acid

Table (4) GC information of Oleic acid

Retention time	18.079
Similarity index	99
Molecular weight	282.468 g/mole
Molecular formula	$C_{18}H_{34}O_2$
Library ID	Cis-9-Octadecenoic acid
Cass number	112-80-1

Anti-bacterial assay of chloroform extract of *T. erecta* flower by disk diffusion method:

The chloroform extract of *T. erecta* flower was screened for its antibacterial activity by disk diffusion method and DMSO used as a negative control against six gram negative bacteria (*Serratia marcescens, A. Spp., E. coli, S. Spp, M. morganii, P. aeruginosa*) and two gram positive bacteria (*S. pneumonia* and *S. aureus*) at concentration of 1000 gm/ml, 500 mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml and 31.25mg/ml of chloroform extract as shown in Table (5) and Figure (6).

The results of Antibacterial activity of *T. erecta* chloroform extract with different bacterial species by disk diffusion method show that the activity is not dose dependent like in well diffusion method.

The most effective concentration was 500 mg/ml and the rest of concentrations give no result against all species of bacteria under study. This method also show that chloroform extract has good antibacterial activity and also confirmed by other studies in the world that show excellent antibacterial activity of chloroform extract of *T. erecta* because it contains essential oil and terpenes with potential antibacterial activity [16,17,18]. In this study two methods were used for studying the antibacterial activity of chloroform extract agar well diffusion and disk diffusion methods. The results of these two methods were differing and the agar well diffusion method show better results, the reason of these different results may related to that disk diffusion method is disk diffusion method allows to simultaneously testing a large number of antimicrobials in a relatively easy and inexpensive manner. However, the results of disk diffusion are considered as qualitative because it can only reveal the susceptibility of antimicrobials against the bacteria tested, which described as susceptible, intermediate, and resistant correlated with diameter of inhibition zone. The major disadvantage of this method is unable to generate the MIC value and difficult to examine the susceptibility of fastidious and slow-growing bacteria. While agar well diffusion method is a quantitative susceptibility testing method. The advantages of agar dilution include the ability to simultaneously test the susceptibility of a number of bacteria in one plate and the ability to test susceptibility testing for fastidious organisms since the agar is able to adequately support the bacteria growth and these differences confirmed by other studies [19].

Table 5: Antibacterial activity of *T. erecta* chloroform extract with different bacterial species measured in millimeter by disk diffusion method.

Bacterial species	Concentrations of chloroform extracts(mg/ml)/inhibition zone in millimeters					Positive control Cefixime	Negative control DMSO
	500	250	125	62.5	31.25		
Serratia marcescens	1	0	0	0	0	0	0
Acinobacter Spp	0	0	0	0	0	0	0
Escherichia coli	1	0	0	0	0	0.5	0
Salmonella Spp	1	0	1	1	0	0.5	0
Morganella morganii	2	0	1	0	0	0	0
Pseudomonas aeruginosa	1	0	1	0	0	0	0
Streptococcus pneumonia	0	0	0	0	0	0	0
Staphylococcus aureus	0	0	0	0	0	1	0

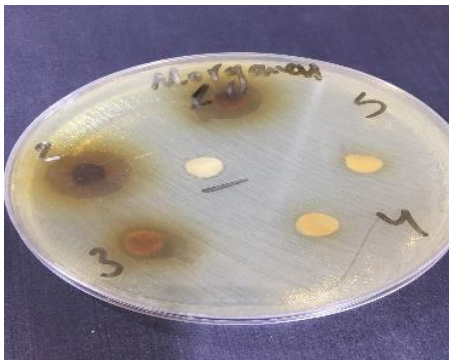

Figure (6): Results of Antibacterial activity of *T. erecta* chloroform extract by disk diffusion method.

Conclusion:

1.A good antibacterial activity of chloroform extract against six species of gram negative (Serratia marcescens, *A. spp., E. coli, S. Spp, M. morganii, P. aerogenosa*) and two were gram positive bacteria (*S. pneumonia and S. aureus*).

2.Agar well diffusion method gives better results than disk diffusion method.

3.*T. erecta* flower considered as an important source of active ingredients with antibacterial activity like stigma sterol, oleic acid and essential oil. Thus, can be a source for developing new natural antibacterial agents.

References:

1- Rayees Ahmad Mir, Surendra Argal and RM Agarwal. Accumulation of Secondary Metabolites and Osmotica in Different Parts of T. erecta L. and its Ecophysiological Relevance. International Journal of Scientific Research and Reviews 2018, 7(1) Suppl., 198-209.

2- Lokesh J Shetty, Farouk M Sakr, Kais Al-Obaidy, Mohammed J Patel, Hidayatullah Shareef. A brief review on medicinal plant T. erecta Linn. Journal of Applied Science 2015, Vol.5, Pages: 091-095

3- Dr. Joseph de Ubaldo. Perspectives on Natural Health: An Anthology of Holistic Medicine 2001.

4- Preeti Verma* and Archana Verma. Evaluation of antibacterial activity of different parts of T. erecta / International journal of pharmacy & life sciences, 2012 3(6): June.

5- Márcia M, Marques M, Selene M, et al. Larvicidal activity of T. erecta against Aedes aegypti. J Am Mosq Control Assoc 2011; 27(2):156-8.

6- Nikkon FM et al. Insecticidal activity of flower of T. erecta against Tribolium castaneum (Herbst). Res J Agr Bio Sci 2009; 5(5):748-53.

7- Ramya R, Bhat S K. Comparative Evaluation of Mycostatic Effect of T. Spp. Indian journal of applied research, Vol 3, Issue 7, July 2013

8- Farjana Nikkon M, Rowshanul Habib, Zahangir Alam Saud and Rezaul Karim M, (Medicinal Plants: Conservation & Sustainable Use) Efficacy Evaluation of Azadirachta Indica, Calotropis Procera, Datura Stramonium and T. Erecta Against Root-Knot Nematodes Meloidogyne Incognita. Pak. J. Bot, 43, 2011, 197-204.

9- L. Barth Reller Melvin Weinstein James H. Jorgensen Mary Jane Ferraro. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices Clinical Infectious Diseases, Volume 49, Issue 11, 1 December 2009, Pages 1749–1755.

10- Brown DF, Kothari D (1975). "Comparison of antibiotic discs from different sources" (PDF). J. Clin. Pathol. 28: 779–83. PMC 475859. PMID 1214010. Retrieved 2013-05-24.

11- Thamer Mutlag Jasim, Mustafa Ghazi Alabbassi and Baydaa Hameed Abdullah, the antimicrobial activities of Pimpinella anisum Extracts against Methicillin Resistant Staphylococcus aureus isolated from Infected Wounds in Tikrit, Iraq. Journal of veterinary medicine 2011/01/01

12- Burt SA, Reinders RD. Antibacterial activity of selected plant essential oils against E. coli O157:H7. Lett Appl Microbiol. 2003;36(3):162-7.
13- Thamer Mouhi Jasiem1, Suhad Faisal Hatem Al-mugdadi2, Ibrahim S. Aljubory1, Qabas Nather Latef, Phytochemical Study and Antibacterial Activity of Crude Alkaloids and Mucilage of Cordia myxa in Iraq, International Journal of Pharmaceutical Sciences Review and Research, 39(1), July – August 2016; Article No. 45, Pages: 232-236
14- Pankaj Gupta and Neeeru Vaudeva In vitro antiplasmodial and antimicrobial potential of T. erecta. Pharmaceutical Biology, 2010,48(11),1218-1223.
15- Kiranmai M and Mohammed Ibrahim. Antibacterial Potential of Different Extracts of T. erecta. International Journal of Pharmacy. 2012,2(1),90-96.
16- A.Kabouche, N. Boutaghane,Z.Kabouche,E.Seguin,F.T illequin,K.Benlabed. Short report Components and antibacterial activity of the roots of Salvia jaminiana. Fitoterapia. July 2005, Volume 76, Issue 5, Pages 450-452
17- FDilika, P. DBremnerJ., J.MMeyer. Antibacterial activity of linoleic and oleic acids isolated from Helichrysum pedunculatum: a plant used during circumcision rites. Fitoterapia. 1 August 2000, Volume 71, Issue 4, Pages 450-452.
18- Pankaj Gupta* and Neeru Vasudeva. Marigold A Potential Ornamental Plant Drug. Hamdard Medicus Vol. 55, No. 1, 2012.
19- iang, Lin, "Comparison of disk diffusion, agar dilution, and broth microdiultion for antimicrobial susceptibility testing of five chitosans" (2011). LSU Master's Theses. 727.