Antibacterial and Wound Healing Activity of Some *Agrimonia eupatoria* Extracts.

Kais Kassim Ghaima*

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Abstract:

The antibacterial activity of some extracts of *A. eupatoria* (aqueous and ethanolic) against some pathogenic bacteria (*Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*) and their activity on wound healing in rats , also the presence of some active compounds in both extracts were detected . The results showed that the ethanolic extract was more effective on inhibiting tested bacteria than the aqueous extract . *P.aeruginosa* was the most resistant bacteria, while highest inhibition zone appeared on *E.coli* (20 mm) .There was a moderate activity against *S.aureus* with inhibition zone 15 mm. by using ethanolic extract (10 mg/ml) . The phytochemical analysis for detection of active compounds revealed the presence of Carbohydrates, Glycosides and Tannins in both extracts, while some of compounds such as Terpenoids and Phenolic compounds (flavonoids) were detected in the ethanolic but not in the aqueous extracts. Prepared ethanolic extract ointment presented obvious activity on wound healing activity in rats in contrast with fucidin ointment and aqueous extract ointment, hence the wound healing was completed in l0 days by using the ethanolic extract ointment, while it was 12 days and 14 days for the aqueous extract ointment and fucidin ointment respectively, in comparison with the untreated wound which needed more than 16 days for healing completion.

Key words: *A. eupatoria* , antibacterial , wound healing .

Introduction :

The plant *A.eupatoria* belongs to the family rosaceae, the name agrimony was derived from (agremone) a word given by the Greeks to plants which were healing to the eyes. The dried aerial parts are used in numerous ways. The leaves when dry retain most of their fragrant odor , as well as the flowers . In some countries the plant is called " a spring drink" or " diet drink" as a purifier of the blood. Internally , it is used in haematuria and diarrhea, externally for wounds and cuts [1]. Restoration of damaged tissue, wound or fracture is an important process, which plays vital role in survival of life. Healing process is a complicated biological process, includes cell regeneration, proliferation, differentiation and synthesis or secretion of different substances [2]. Plants and their extracts have immense potential for the management and treatment of wounds. The phyto-medicines for wound healing are not only cheap and affordable but are also purportedly safe as hypersensitive reactions are rarely encountered with use of these agents [3]. Different parts of plants used for wound healing contain some active components that are antimicrobial and nutritive in function [4]. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are most common pathogens which infect the skin and *Escherichia coli* which is an opportunistic pathogen at the site of cut wounds[5]. Main stream medicine

*Institute of Genetic Engineering and Biotechnology for Postgraduate Studies , University of Baghdad.

is increasingly receptive of the use of antimicrobial and other drugs derived from plants as traditional antibiotics become ineffective and because of the rapid rate of plant species extinction. Herbs and spices are generally considered safe and proved to be effective against certain ailments [6]. The main objectives of this study were evaluation of antibacterial activity of *A. eupatoria* plant extracts (aqueous and ethanolic) against some of pathogenic bacteria and there's activity on wound healing in rats , also detection of some active compounds in both extracts in order to justify therapeutic using of the plant.

Materials and Methods

Plant materials

The plant *A. eupatoria* was collected from the markets of Sulaimania in North of Iraq. The plant was identified and autnenticated by Prof. Dr. Ali Al-mosawy, Department of Biology, College of Science, University of Baghdad.

Preparation of plant extracts

a- Aqueous extract (Hot- water extraction)

Exactly 20gm of dry areal parts soaked in 100ml of hot distilled water of 80 ^{o}C incubated on shaker water bath at 150 rpm for 24 hr. to allow a proper extraction and it was filtered through filter paper(Whatman No.1) after which the extract was obtained , air dried and stored at 4 ^{o}C until required [7].

b- Crude ethanolic extraction

Air dried sample (20 gm) was soaked in 100 ml of 95 % ethanol, and incubated on a shaker at 150 rpm for 24 hr. at 30 ^{o}C . The extract was filtered through filter paper (Whatman No.1) which was impregnated with the same solvent. The ethanol was concentrated to near dryness under reduced pressure below 40 ^{o}C using Rotary evaporator. The amount of the concentrate extract was noted down. The extracts were diluted to 20 mg/ml with 10 % dimethyl sulfoxide (DMS) solution and stored in air tight glass bottles at 4 ^{o}C till further use [8].

Microorganisms and media

The bacterial isolates *S.aureus* and *P. aeruginosa* were collected from burn patients, while *E.coli* was isolated from gastrointestinal infection. All the bacteria were obtained, as clinical isolates, from Al- Yarmook teaching hospital in Baghdad. Bacterial cultures were maintained on Nutrient agar (NA) slops. They were subcultured monthly and subsequently stored at 4 ^{o}C.

Culture preparation

A loopfull of 24 hr. surface growth on a NA slope of each bacterial isolate was transferred individually to 5 ml of Brain heart infusion broth (pH 7.6) and incubated at 37 ^{o}C for 24 hr. bacterial cells were collected by centrifugation at 3000 rpm for 15 min, washed twice and resuspended in 0.1 % peptone water. Turbidity was adjusted to match that of as McFarland standard (10^{8} cell/ml). Then 1:10 dilution of the cell suspension was performed to give an inoculum concentration of 10^{7}(cell/ml).

Antibacterial activity test of extracts (*in vitro*) using agar diffusion assay method.

A 0.2 ml volume of the standard inoculums (10^{7} cell/ml) of the test bacterial isolate was spread on Mueller Hinton Agar(MHA) with a sterile glass rod spreader and allowed to dry. Then, 6mm. diameter wells were bored using cork borer in the MHA. Plant extracts (1,5 and 10 mg/ml concentration) were introduced into each well and allowed to stand for 1 hr. at room temperature to diffuse the plant extracts into medium before incubation at 37 ^{o}C for 24 hr. The Inhibition zone diameter (ISD) was measured by transparent ruler to nearest mm [9]. Ciprofloxacin (10 mg/ml) (Oxoid) was used as

positive control . Inhibition zone with diameter less than 12 mm. were considered as having no antibacterial activity , diameter between 12 and 16 mm. were considered moderately active , and those with > 16 mm. were considered highly active [10].

Phytochemical analysis

The Identification tests were done to find the presence of the active chemical constituents such as , Alkaloids, Carbohydrates, Steroids, Terpenoids, Glycosides , Saponins ,Phenolic compounds (flavonoides) and Tannins by the procedure as described by Siddiqui and Ali (1997) [11].

Wound healing activity of extracts (*in vivo*)

The wound evaluation model of Arzi *et al*., (2003) [12] was adopted with some modification.

Ointment preparation

Polyethylene glycol was used as a water soluble base to prepare ointments of *A. eupatoria* extracts in 10 % (w/w) concentration (12)

Experimental animals

Male, Wister albino rats 4 weeks of age weighing between 80- 100 gm were obtained , from the College of Veterinary Medicine, University of Baghdad . The rats were housed in a ventilated animal house before and after surgery. The holding room was illuminated with 12 hr. light/ dark cycles. Room temperature was between 30-35^{o}C.

***In vivo* wound healing activity of *A. euputoria* extracts (12)**

Full thickness wounds were made in the skin of the tested animals, hair of lower back and right flank of animals were fully shaved. Rats were lightly anaesthetized by inhalation using halothane. The animals were held in standard crouching position, and the mobile skin of flank was gently stretched and held by fingers. A metal circular object measuring 1 cm. in diameter was placed on stretched skin and an outline of the object was traced on the skin using a fine tipped pen. The wound was made by excising the skin within the border of the object to level of loose subcutaneous tissue, using sterile forceps and scalpel blade the artificial wounds were circular with diameter of 1 cm. . The first day of the experiment was regarded as day zero.

Animals were divided into four groups, each containing three animals.

Group 1: Untreated control group, wounds were left without treatment.

Group 2: Wounds of these animals were treated topically with Fucidin ointment every 24 hr. as standard healing agent from first day.

Group 3: Wounds of these animals were treated topically with *A. eupatoria* ethanolic extract ointment (10 %) every 24 hr. starting from first day.

Group 4: as group 3 but the extract was aqueous extract.

Evaluation method of wound healing percentage

In order to determine the rate of wound healing every 24 hr., each animal was held in the standard crouching position and two diameters of the wound circle (horizontal and vertical) were measured using a transparent ruler. Measurement errors were minimized by repeating each measurement three times at the same moment and using an average of the calculations. The area of the wound in day zero considered as 100 % and wound areas on subsequent days were compared with the wound on day zero. Healing percentage in a certain day was the difference between the initial wound (in zero days) and healing wound on that certain day.

RESULTS AND DISCUSSION

The results of antibacterial activity of *A. eupatoria* extracts on the pathogenic bacterial isolates (*S. aureus*, *P. aeruginosa*

and E. coli) are shown in table (1).

Table 1. Antibacterial activity of *A. eupatoria* extracts against some of pathogenic bacteria.

No.	Pathogenic bacteria	Diameter of inhibition zone (mm)						
		Concentration of ethanolic extract (mg/ml)			Concentration of aqueous extract (mg/ml)			Ciprofloxacin
		1	5	10	1	5	10	10 mg/ml
1.	*S. aureus*	N.I	10	15	N.I	4	10	18
2.	*P. aeruginosa*	N.I	4	12	N.I	N.I	N.I	16
3.	*E. coli*	8	10	20	4	8	12	25

* Data are means of two replications.

** N.I: No Inhibition.

The results indicate that the ethanolic extract of *A. eupatoria* was more effective on the bacterial isolates than aqueous extract, this may explaine by the fact that the antimicrobial substance in the agrimony extracts mainly phenolic compounds are destroyed by heat from hot water which might have raised the temperature of the extracts inactivating them [13,14]. Highest inhibition was noticed against *E. coli* with ethanolic extract at concentration of 10 mg/ml (with highest inhibition zone 20 mm). *P. aeruginosa* was more resistant than other bacteria, where moderate antibacterial activity was observed by ethanolic extract at concentration of 10 mg/ml. Also the results exhibited that ethanolic extract (10 mg/ ml) was moderately active on *S. aureus*, while no significant activity by aqueous extract at the same concentration. It's obvious that the antibiotic ciprofloxacin was more active on bacteria than extracts, this may be due to high activity of antibiotic on gram positive and gram negative bacteria by inhibition DNA synthysis. The topical application of plants used in folklore at the wound healing activity which may be due to antibacterial activity of the chemical constituents present in the crude extract and the delays in healing process directly promote the microbial infection [15]. Tannins and other polyphenols inhibit the microbial growth and have the ability to inactivate the microbial adhesion, enzymes and cell envelope transport proteins [16]. Flavonoids belong to polyphenols have several biological activity, such as antiinflammatory, antiulcer, anti cancer, antiviral, antibacterial and antispasmodic have been attributed to these compounds [17].Phytochemical analysis of ethanolic and aqueous extracts of *A. eupatoria* (Table .2) revealed the presence of Carbohydrates,

Glycosides and Tannins in both extracts, while some compounds such as Terpenoids and phenolic compounds (flavonoids) were detected in ethanolic extract but not in the aqueous one, because they were highly dissolved in alcohol. Agrimony contains up to 3.13 % Condensed tannins, glycosidal bitters, nicotinic acid, volatile oil, around 20 % polysaccharides, silica, flavonoids, mucilage, vitamins B and K and triterpenoids [18].

Table 2. Chemical constituents of *A. eupatoria* extracts.

No.	Chemical test	The type of extracts	
		ethanolic	aqueous
1.	Alkaloids	-ve	-ve
2.	Carbohydrates	+ve	+ve
3.	Steroids	-ve	-ve
4.	Proteins and amino acids	-ve	-ve
5.	Terpenoids	+ve	-ve
6.	Glycosides	+ve	+ve
7.	Saponins	-ve	-ve
8.	Phenolic compounds	+ve	-ve
9.	Tannins	+ve	+ve

+ve = detected -ve = not detected

The wound healing activity of *A. eupatoria* extracts (ethanolic and aqueous) on rats are summarized in table (3) and figure (1).

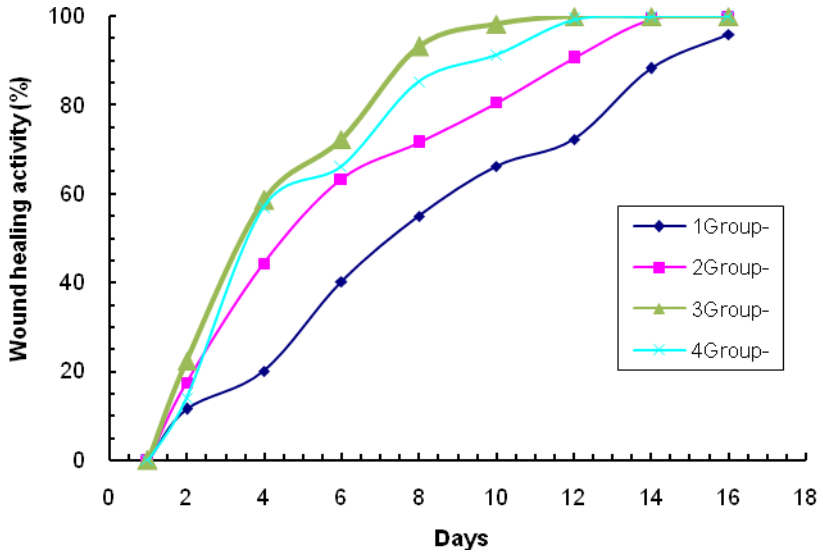

Figure 1. The wound healing activity of *A. eupatoria* extracts (ethanolic and aqueous) on rats :(the percentage represent mean of three rats)

Group 1: Untreated control group, wounds were left without treatment.

Group 2: Wounds of these animals were treated topically with Fucidin ointment every 24 hr. as standard healing agent from first day.

Group 3: Wounds of these animals were treated topically with *A. eupatoria* ethanolic extract ointment (10 %) every 24 hr. starting from first day.

Group 4: as group 3 but the extract was aqueous extract

Table 3. Percentage of wound healing activity (%) of *A. eupatoria*_extracts on rats.

Group No. / Day No.	Group 1 (wound. only)			Group 2 (wound+ fucidin)			Group 3 (wound+ 10% PEG ethanolic)			Group 4 (wound+ 10% PEG aqueous)		
	Rat 1	**Rat 2**	**Rat 3**	**Rat 1**	**Rat 2**	**Rat 3**	**Rat 1**	**Rat 2**	**Rat 3**	**Rat 1**	**Rat 2**	**Rat 3**
First day	0	0	0	0	0	0	0	0	0	0	0	0
Second day	0	13	10	15	0	20	20	27	26	22	18	12
4^{th} day	20	24	16	43	44	46	59	65	52	58	62	51
6^{th} day	41	48	36	63	57	60	75	67	73	62	70	66
8^{th} day	54	62	49	72	74	69	96	91	93	81	89	86
10^{th} day	65	76	58	74	83	80	100	95	100	89	95	93
12^{th} day	71	79	67	91	92	89	100	100	100	100	100	98
14^{th} day	88	90	81	98	100	100	100	100	100	100	100	100
16^{th} day	96	100	92	100	100	100	100	100	100	100	100	100

In the group 1 (untreated control group), healing was completed in 16 days, in the second group (wound were treated with fucidin ointment) more than 14 days were required for the completion of healing. The healing period was reduced to 10 days in the third group when the wound treated with PEG containing *A. eupatoria* ethanolic extract 10% ointment, while it was reduced to 12 days in the fourth group when the wound treated with PEG containing *A. eupatoria* aqueous extract 10 % ointment.

On 10th post wounds days, group 1 animals showed 66.3% of healing (which may be due to self immunity of the animals), whereas fucidin treated animals showed 80.6% of healing. On the other hand; the group treated with ethanol extract ointment showed 98.3% of healing to be obvious when compared with the control.On the basis of the results obtained in the present investigation, it is possible to conclude that the ointment of the ethanolic extract of *A. eupatoria* has significant wound healing property appeared to be due to the presence of its active principles, which accelerates the healing process, this effect can be attributed to the high silica content of *A. eupatoria* [19]Tannins are astringent and antimicrobial in property, hence it can be inferred that the wound healing activity is due partly to it's tannin and flavonoids content, which seems to be responsible for wound contraction and increased rate of epithelization [20]. Significant activity was observed for polyphenolic compounds of *A. eupatoria* with recognized anti-inflammatory properties such as procyanidins and quercetin [21]. The difference in effect between ethanolic and aqueous extracts on wound healing may be due to the absence of some active constituents from aqueous extract such as polyphenolic compounds and terpenoids, which play an important role in the antioxidant, anti- inflammatory and anti-bacterial activity [22]. Thang *et al.* (2001) [23]mentioned that in cutaneous tissue repair, oxidants and antioxidants play important roles, the administration of antioxidants or free radical scavengers is helpful, in order to enhance the healing process. The aerial parts of Agrimony are used as infusions (aqueous and alcoholic extracts) in traditional medicine, for their anti-inflammatory, astringent and diuretic properties [1].

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الفعالية المضادة للبكتريا والتئام الجروح لبعض مستخلصات نبات الغافث *Agrimonia eupatoria*

قيس قاسم غيمة*

*معهد الهندسة الوراثية واالتقنيات الاحيائية للدراسات العليا/ جامعة بغداد .

الخلاصة :

بُحثت الفعالية المضادة للبكتريا لبعض مستخلصات نبات الغافث (المائي والايثانولي) تجاه بعض البكتريا الممرضة (*Escherichia coli, Pseudomonas aeruginosa , Staphylococcus aureus*)وفعاليتها في التئام الجروح في الجرذان ، كما كشف عن وجود بعض المركبات الفعالة في المستخلصين. اظهرت النتائج ان مستخلص الغافث الايثانولي اكثر تأثيراً في تثبيط البكتريا قيد الاختبار من المستخلص المائي ، وان بكتريا *P.aeruginosa* كانت الأكثر مقاومة للمستخلصات في حين ان اكبر منطقة تثبيط لوحظت مع بكتريا *E.coli* (20 ملمتر) ، وان هنالك فعالية متوسطة للمستخلص الأيثانولي (10 ملغرام/ مللتر) تجاه بكتريا *S.aureus* وبقطر منطقة تثبيط 15 ملمتر . اختبارات الكشف الكيمياوي النوعي عن بعض المركبات الفعالة في المستخلصين اظهر وجود مركبات الكاربوهيدرات ، الكلايكوسيدات والتانينات في كلا المستخلصين بينما بعض المركبات مثل التربينويدات والمركبات الفينولية (الفلافونويدات) وجدت في المستخلص الكحولي دون المائي. اعطى مرهم مستخلص الايثانول فعالية واضحة في التئام الجروح في الجرذان مقارنة بمرهم الفيوسيدين ومرهم المستخلص المائي، حيث ان التئام الجروح قد اكتمل خلال 10 ايام باستخدام مرهم المستخلص الأيثانولي بينما كانت 12 يوماً و14 يوماً لمرهم المستخلص المائي ومرهم الفيوسيدين على التوالي ، وبالمقارنة مع الجروح غير المعاملة التي احتاجت الى اكثر من 16 يوماً لغرض اكتمال الالتئام.