RESEARCH ARTICLE

Evaluation of Sperm Head Abnormality Traits and Anti-bacterial Properties of *Achillea millefolium* Methanolic Extract on Methotrexate-induced Albino Male Mice

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ABSTRACT

Plants have been used by people from prehistoric times to get rid of suffering and curing ailments. Plants are of the important sources of medicine, and a large number of drugs in use are derived from plants. Yarrow, a member of the aster family, is closely related to chrysanthemums and chamomile. Yarrow (*Achillea millefolium*) was named after Achilles, the Greek mythical figure who used it to stop the bleeding wounds of his soldiers. Decoctions have been used to treat inflammations, such as hemorrhoids and headaches. Yarrow contains flavonoids (plant-based chemicals) that increase saliva and stomach acid, helping to improve digestion. Phenolic acids, such as caffeic acid and salicylic acid, are components of the bioactive compounds present in *Achillea millefolium* that have anti-inflammatory and anti-bacterial properties. This study was designed to assess the fertility and anti-bacterial activity of Yarrow methanolic extract through in vivo (albino male mice) and in vitro (antibacterial activity) studies. Results indicated that higher doses of the plant increased sperm head abnormality, and this lead to decreased fertility in addition to that antibacterial activity was dose-dependent manner. Conclusions: Yarrow contains flavonoids and other chemical constituents that have these effects.

Keywords: *Achillea millefolium*, Antibacterial activity, Sperm head abnormality.

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INTRODUCTION

Herbal medicine is a major component of all indigenous people's traditional medicine and a common element in Ayurveda, Homoeopathic, Naturopathic, Traditional Arabic, Oriental, and Native American, Indian medicine.[1] Scientific investigations confirmed these medicinal potentials, which has been presented in vitro and in vivo pieces of evidence. Those medicinal plants or their secondary metabolites have shown different biological effects with a wide range of pharmacological properties.[2] Among these, plants are species of the genus *Achillea*, which belongs to the family Asteraceae and includes about 85 species of trees and herbs. The genus *Achillea* found in the Northern hemisphere, mostly in Europe and Asia.[3] Generally, yarrow species are used against digestive problems, liver and gallbladder conditions, menstrual irregularities, cramps, inflammation, fever; they have a wound-healing effect and increase urine flow.[4] Yarrow (*Achillea millefolium*) flourishes in a sunny and warm habitat and is frequently found in meadows and along roadsides, as well as on dry, sunny slopes.[5] The flowers, essential oil, leaves, as well as aerial parts are useful in some way, or the other.[6] *Achillea millefolium* has different names Green arrow, Milfoil, Noble yarrow, Solder's woundwort and others.[7] The volatile oils presented in plant work as antibacterial, anti-inflammatory and diuretic agents.[8] The tannins are aggressive astringents, alkaloids are both hypotensive and hypoglycemic. Yarrow (*Achillea millefolium*) even has coumarin in its cells, which works as an anti-thrombotic to reduce high blood pressure.[9] The bitter compounds that the tongue detects are due to flavonoids such as saponins and unpleasant tasting but powerful alkaloids like achilleine, trigonelline and betonicine.[10]

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METHODS

Plant Collection and Identification

The aerial parts of plants were supplied from the local markets in (Baghdad\Iraq) during Sep., 2018; and recognized by Dr. Khulood W. AL-Samarraei (College of Biotechnology\ Al-Nahrain University)

Preparation of Plant Extract (Soxhlet extraction or continuous hot extraction)

In this method, a finely ground plant is placed in a porous bag or "thimble" made from a strong filter paper that is placed in the thimble chamber of the Soxhlet apparatus. Extraction solvent (80% methanol) is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser, and drip back. When the liquid content reaches the siphon arm, it contents emptied into the bottom flask again, and the process is continued. The extract solution was concentrated to dryness under reduced pressure in a rotary evaporator to yield dried crude extract, which was frozen at -20°C until use to prepare the required doses and concentrations.11

Doses and Concentrations

In albino male mice, two doses of the extract were tested (100 and 200 mg/kg). The selection of doses was based on a previous investigation, in which two doses of alcoholic extract of *Achillea millefolium* flower studies on the fertility of rats.12

In an antibacterial study, three concentrations of the extract were tested (100, 200, and 300 mg\mL). To prepare these doses and concentrations, the dried methanolic extract was dissolved in a few drops of DMSO (Dimethyl sulfoxide) and then diluted further with distilled water to the required volume of the dose. Then filtered by Millipore filter paper (0.22 mm) and stored at 4°C until used.

Laboratory Animals

Albino male mice (*Mus musculus*) were the laboratory animals, which were used to carry out the investigations of the present study. They were obtained from the Biotechnology Research Centre (Al-Nahrain University). Their age range was 8-9 weeks, and their weight was 23–27 grams. They were caged in the animal house of the supplier, in which the temperature was 23-26°C, and a light: dark periods of 10:14 hours/day. The animals had free excess to food (standard pellets) and drinking water (*ad libitium*).

Experimental Design

For *in vivo* studied, the mice groups were divided into four groups which were:

- **Group I**: The animals were treated with distilled water (negative control = 4 animals).
- **Group II**: The animals were treated with the first dose of plant extract (100 mg\kg = 4 animals)
- **Group III**: The animals were treated with the second dose of plant extract (200 mg\kg = 4 animals)
- **Group IV**: The animals were treated with (methotrexate drug 40 mg\kg = 4 animals) (positive control)

The tested materials were injected intraperitoneally as a single dose (0.1 mL) per day and for 7 days. Then the mice were sacrificed on day 8 for laboratory assessments. The total number of mice was 16 animals.

Sperm-head Abnormality Assay (SHA)

The procedure of Wyrobek, A. J. *et al.,*[13] was used in which mouse was sacrificed by cervical dislocation and then dissected to obtain the epididymis, which was collected in as Petri-dish containing 5 mL of normal saline which forceps and a scalpel to free the spermatozoa. The spermatozoa-containing saline tube was centrifuged (1000 rpm) for 10 minutes, and the spermatozoa deposit was gently suspended in 1 mL of normal saline. A thin smear of the suspension was made on a clean slide, which was stained with eosin for 5 minutes and examined under oil immersion lens (100X), and at least 1000 spermatozoa were inspected for the morphology of their heads. The sperm-head abnormality (SHA) index was scored using the following equation:

$$\text{SHA Index}\,(\%) = \left(\frac{\text{Numbers of Spermatozoae with Abnormal Head}}{\text{Total Count}}\right)$$

IN VITRO STUDY FOR DETERMINATION OF ANTIBACTERIAL ACTIVITY

Preparation of media

Muller Hinton agar 38g was suspended in 1000 mL D.W, heated to boiling to dissolve the medium completely, then sterilize by autoclaving at 15 Ibs pressure (121c) for 15 min, mix well before pouring. The agar was poured to a depth of 3–4 mm, after solidification, plates were kept at 4°C to provide a firm surface for wells making which were filled with 100 microliters of *Achillea millefolium*.[14]

Determination of Anti-bacterial activity

Single colonies grown on nutrient agar for (18-24)hours were transferred to a tube containing 5mL of normal saline and mixed well by vortex, then bacterial growth was compared with McFarland tube No.0.5 turbidity standard solution, which was equivalent to a bacterial inoculum concentration of ($1.5*10^{\wedge 8}$) cell/mL.[15] A touch of bacterial culture from normal saline was transferred to Muller Hinton agar by using a cotton swab and streaked three times by rotating the plate approximately 60º between streaking to ensure even distribution of the inoculums; the inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture.[16] Then, sterilized pauster pipette was used for making wells (the wells were arranged so as to avoid the development of overlapping of inhibition zones) which were filled with 100 µL of (*Achillea millefolium*) by using 100, 200 and 300 mg\ mL and the plates were incubated at 37ºC for 18-24 hours.[17] After incubation, inhibition zone were measured by the ruler to a determination of their diameters in millimeters; then the results were recorded.[18]

RESULTS

Sperm-head Abnormality Assay

Different sperm-head abnormalities were observed as a result of treatment with the MTX drug (Figure 1: A and B), which

was associated with a significant increase in sperm-head abnormalities (68.76%) as compared to negative controls (60%). The two doses of plant methanolic extract (100 and 200 mg/mL) had able to modulate the effects of drugs on sperm head abnormality in a dose-dependent manner.

Antibacterial activity of *Achillea millefolium* methanolic extract against different pathogenic bacteria isolated from UTI infections.

Antibacterial activity of *Achillea millefolium* aerial parts methanolic extract against different pathogenic bacteria isolated from UTI infections, which were supplied from the College of Biotechnology\Al-Nahrain University, was detected. Different concentrations of *Achillea millefolium* aerial parts methanolic extract were used. Results reveals that *Achillea millefolium* aerial parts methanolic extract at all concentrations (100,200 and 300 mg/mL) have moderate antibacterial activity against some pathogenic bacteria (*Staph aureus* and *Pseudomonas aeruginosa)* in which the diameter of zone of inhibition range from (8-15)mm for *Staph aureus* and from (9–15) for *Pseudomonas aeruginosa* as shown in Figure 1 and 2 and the inhibition activity was concentrations dependent manner (increased the concentrations of *Achillea millefolium* aerial parts methanolic extract lead to increased

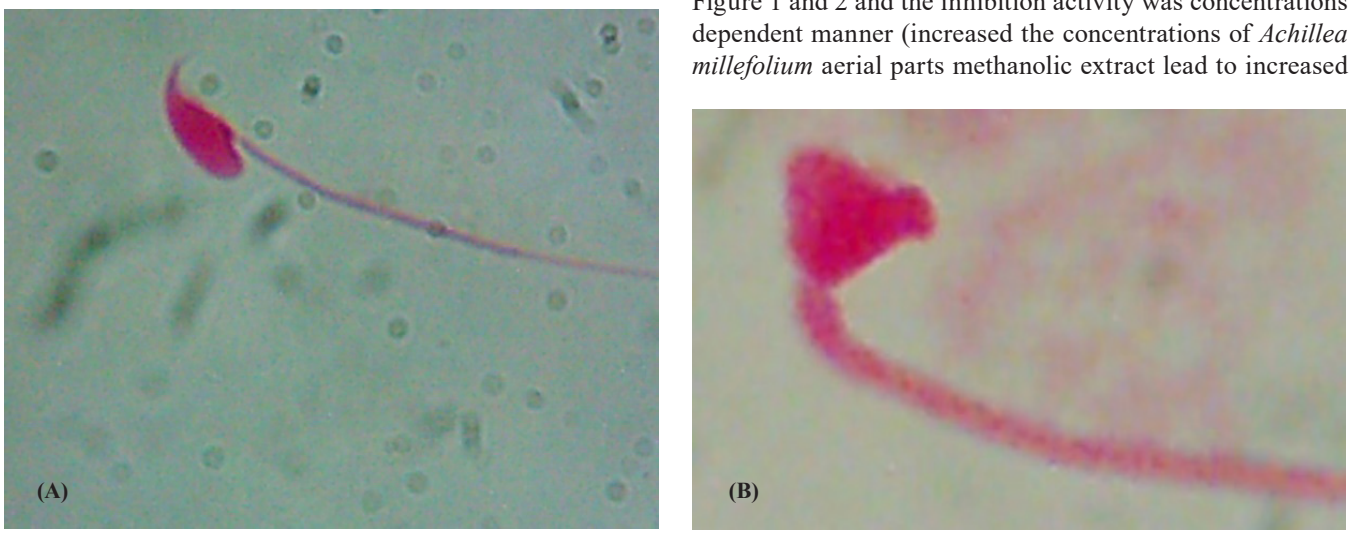

Figure 1: Normal (A) and abnormal (B) sperm heads in mice treated with MTX drug (100X)

Figure 1: Antibacterial activity of *Achillea millefolium* against *Staph aureus* isolate.

Figure 2: Antibacterial activity of *Achillea millefolium* against *Pseudomonas aeruginosa* isolate.

Table 1: Sperm-head abnormalities (mean ± standard error) in albino male mice treated with methanol extract of *Achillea millefolium* aerial parts, distilled water (negative control), and MTX drug (positive control).

Groups	*Dose (mg/kg)*	*Mean ± Standard Error (%)*
Positive Control (MTX Drug)	40	68.76 ± 0.84 a
Negative Control (Distilled Water)	0.00	60.00 ± 0.63 b
Achillea millefolium Methanolic Extract	100	31.33 ± 7.54 d
	200	52.76 ± 8.87 c

Different letters: Significant difference ($p \leq 0.05$) between means of columns.

Table 2: Antibacterial activity of the *achillea millefolium* methanolic extract on growth of *Staph aureus* and *Pseudomonas aeruginosa* isolates.

	Concentrations of Achillea methanolic extract (mg\ml) and Mean of Zone of inhibition (mm)		
Bacteria Spp.	*100 mg\mL*	*200 mg\mL*	*300 mg\mL*
SA1	7	13	15
SA2	10	8	12
PS1	9	-	11
PS2	9	-	15

SA: *Staph aureus*; Ps: *Pseudomonas aeruginosa* (each SPP. Contain 3 strains).

the zone of inhibition).

While *Escherichia coli* and *Streptococcus* both were resistance to the *Achillea millefolium* methanolic extract at all concentrations used.

DISCUSSION

The results of genetic evaluations showed that treatment with *Achillea millefolium* methanolic extract was associated with a significant reduction in sperm head abnormalities, and such effect was dependent on dose.[19] Numbers of studies have been conducted in regard to the antioxidant activity of *Achillea millefolium*, and in general their findings are in agreement with the present results. Some scientists founded that *Achillea millefolium* contained active compounds like alkaloids, glycosides, flavonoids, tannins, resins, coumarins, terpenes, and saponins which could act as anti-oxidant activity and was free radical scavengers[20] while[21] confirmed that these compounds are effective in scavenging free radicals. Such effects can be achieved through several metabolic pathways; for instances, inhibit the formation of free radicals, suppress chain initiation and/or breaking chain propagation reaction, increasing the activity of detoxifying enzymes such as glutathione transferase (GST) and superoxide dismutase (SOD) and *de novo* anti-oxidant and adaptation where the signal for the production and reaction of free radicals formation and transport of the anti-oxidant to the right site.[22]

Our results agree with the results of 12 who showed that alcoholic extract of *Achilea mellefolium* flowers in higher doses could decrease fertility in male rats. Another in vitro study was carried out on the effect of aqueous and ethanolic of extracts on some sperm functions including sperm motility, viability and morphologically showed that *Achilea mellefolium* at high doses increased abnormal sperms' percentage both after one minute, and 30 minutes of incubation and reduction in all sperm functions and this agreed with our results.[23]

The results indicated above explain the antibacterial activity of the plant extract due to containing a high flavonoids content.[24] Flavonoids exhibited strong antibacterial activities, and these activities may be linked with the presence of essential oil compounds in the extract.[25] Our results agree with the results of[26] whose found that *Achillea millefolium* contains essential oil compounds which effective against *Staph aureus, Pseudomonas aeruginosa*, and *Escherichia coli* in a varying degree[27] showed that essential oil represented antimicrobial activity on a different pathogenic organism. These scientists showed that the inhibition zones from the essential oils were generated against *Staph aureus* by the diameters of inhibition zone (8 mm), and there was no inhibition zone generated against *Escherichia coli*.

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