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Assessments of Immunological Activity of *Achillea Millefolium* Methanolic Extract on Albino Male Mice

Ruqaya Mohammed Al-Ezzy[1], Rafal S. A. Al Anee[2] and Niran A. Ibrahim[3]

1. Departement of Molecular and Medical biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq

2. College of Pharmacy, Al-Nahrain University, Baghdad, Iraq

3. College of Education for Pure Sciences (Ibn Al-Haitham), University of Baghdad, Baghdad, Iraq

Abstract: *Achillea millefolium* (Asteraceae) is a permanent herb highly recognized in traditional medicine for its anti-oxidant and anti-inflammation properties. However, studies on phytochemical constituents of *A. millefolium* underlying these properties are scarce. The present work focuses on examining the effect of methanol extract of *A. millefolium L.* on total and differential blood cells account on albino male mice. The results showed the methanol extract increased the account of lymphocyte, and monocyte cells, and total account as well as this extract showed high decrease in the oxidative stress of MTX after the interfere between the extract and MTX due to increase in the leucocyte cells compared with controls. Concluded from these results that methanol extract of *A. millefolium* has ability enhancement in leucocyte cells in the blood and it has detoxification effect of MTX.

Key words: *Achillea millefolium*, white blood cells, methanol extract, total account, differential account and albino male mice.

1. Introduction

Plants are the important sources of medicine and a large numbers of drugs which used and derived from plants. The therapeutic uses of plant are safe and economical and effective as their ease of availability [1]. *Achillea millefolium L.* (Asteraceae) is a plant known as Yarrow; it is widely used in both folk and official medicine [2]. Greek mythical figure used it to stop the bleeding wounds of his soldiers. Decoctions have been used to treat inflammations, such as hemorrhoids, and headaches [3]. This traditional medicine plant is used to treat respiratory infections, fever, rheumatic pains and has a mild stimulant effect, and has been used as a snuff [4], as well as it contains flavonoids (plant-based chemicals) that increase saliva and stomach acid, improve digestion, also may relax smooth muscle in the intestine and uterus, which can relieve stomach and menstrual cramps [5]. While the essential oil which is extracted from flower displays different chemical profiles based on geographical origin, but the major constituents remains as: azulene, cineol, borneol, pinenes and camphor. This essential oil has many therapeutic properties such an anti-inflammatory, in chest rubs for colds, influenza and is treated various allergic mucus problems, including hay fever [6]. But the fresh juice or chilled extracts from dried herb were applied to stanch blood release; whereas fresh chopped leaves were used to heal bruises, ulcers and wounds [7]. Tested *n*-hexane extracts showed anti-fungal and herbicidal activity. Zinc is one of the most important microelement in yarrow used in a skin inflammation treatment, as lichens and acne [8]. Also this plant used in formulas of emulsions and creams for greasy and mixed skin and shampoos for greasy hair and against hair loss. Beauty mask made of the herb has a softening, cleaning, anti-oiling and bleaching [9]. The purpose of this research is study the effect of methanol extract of *Achillea millefolium* on peripheral blood cells in albino male mice.

Corresponding author: Ruqaya Mohammed Al-Ezzy, Ph.D., Lecturer, research fields: biotechnology.

2. Materials and Methods

2.1 Methods

2.1.1 Plant Collection and Identification

The areal parts of plant were collected from the local markets during September (2016), which had been identified previously by National Herbarium of Iraq.

2.1.2 Preparation of Plant Extract

The extract was prepared according to method presented by Ref. [10] in which fifty grams powder was soaked in (1L) of 80% methanol, for one hour in sonication, then 24 hours of stirring the mixture, was then filtered using a Buchner funnel under vacuum pressure repeated two times. The filtered solution was dried using a rotary evaporator under vacuum, then dry freezing weighted and the dark brown sticky residue. The extract was stored under sterile conditions, protected from light in a dry and cool place at -20°C until use.

2.1.3 Laboratory Animals

Albino male mice aged 6-8 weeks and weighted 23-25 gm were purchased from Biotechnology Research Center\Al-Nahrain University\Baghdad\Iraq. Four animals were housed per cage with *ad libitum* access to water and food pellets. They were divided into six groups (details of these groups are given in the section of experimental design).

2.1.4 Absolute and Differential Counts of WBC (white blood cells)

Manual total WBC counts were determined using hemacytometer chamber after lysis of red blood cells with 2% acetic acid. For differential counting, blood smears were fixed with 100% methanol for 5 minutes and then stained with Lieshmann stain [11]. Lymphocytes, neutrophils, eosinophils, basophils and monocytes were identified by their staining properties under optical microscopy. A total of 200 cells were counted and expressed as the percent of the specific cell type which then was converted to absolute counts utilizing the total WBC counts previously determined [12].

2.2 Experimental Design

2.2.1 The First Experiment

The first experiment was designed to assess the immunological (total and absolute counts of WBCs) of two doses (100 and 200 mg/kg) of the plant extract, as well as, the drug methatroxate (40 mg\kg). In these investigated parameters, a single dose/day (0.1 mL) of the tested material was injected intraperetoially for seven days, and in day 8, the animals were sacrificed to carry out laboratory assessments. The total number of animals in this experiment was 16 mice, which were divided into four groups as explained in Table 1.

2.2.2 The Second Experiment

This experiment was designed to assess interactions between both doses (100 and 200 mg/kg of plant extract) and the drug methotrexate through post treatment with the plant extract, in which the animals were injected with methotrexate in day 1, while in days 2-7, they were injected with the plant extract (single dose/day). The animals were sacrificed in day 8 for laboratory assessments. Details of these groups are summarized in Table 2.

2.3 Statistical Analysis

Data were presented as mean ± standard error (S.E.) for in vivo studies. To get such data, the individual values

Table 1 Laboratory tests and number of animals in the investigated groups of experiment number one.

Groups	Tested material	Dose (mg/kg)	Laboratory tests and number of animals
			TC and DC
Group I	Distilled H_2O		4
Group II	Methotrexate	40	4
Group III	*A. millefolium* methanol	100	4
and Group IV	Extract	200	4
Total number of animals			16

TC: Total counts of leucocytes; DC: Differential counts of leucocytes.

Table 2 Laboratory tests and number of animals in the investigated groups of the second experiment.

Type of treatments	Type of interaction	Laboratory tests and number of animals
		TC and DC
Post-treatment Methanol extract	Methotrexate + distilled water	4
	Methotrexate + plant extract (100 mg/kg)	4
	Methotrexate + plant extract (200 mg/kg)	4
Numbers of animals		12

TC: Total counts of leucocytes; DC: Differential counts of leucocytes.

were tabulated in a sheet of the statistical programme GraphPad Prism version 5.01 (GrapgPad software, Inc., La Jolla, CA, USA), and Statistical analysis system-SPSS version 14 was used to effect different actors in study parameters. The difference between means was assessed by Duncan's test, in which $P \leq 0.05$ was considered significant.

3. Results

3.1 Total Acount

The MTX drug was effective in a significant reduction of leucocytes (1,532.33 cell/cu.mm.blood) as compared to distilled water (7,500 cell/cu.mm.blood) negative controls (Table 3).

The first dose of methanol extract (100 mg/ml) increased the count of leucocytes (9,300 cell/cu.mm.blood), while the next dose (200 mg/ml) reduced such count (6,174.00 cell/cu.mm.blood,) as compared to the corresponding negative control (7,500 cell/cu.mm.blood). However, these deviations were statistically significant.

The interaction groups 1 and 2 showed a significant increase in the total account of lymphocyte (5,333.33 and 5,866.66 cell/cu.mm.blood, respectively) as compared to the positive control.

3.2 Lymphocyte

The negative control showed leucocyte counts of 7,130.33 cell/cu.mm.blood, which was significantly higher than the count that was observed in the positive control (1,148.00 cell/cu.mm.blood) (Table 4).

The first dose of methanol extract (100 mg/mL) was significantly effective in increasing the count of lymphocytes (8,904 cell/cu.mm.blood), while the next dose (200 mg/ml) contributed in a significant reduction of such count (5,800 cell/cu.mm.blood) as compared to the corresponding negative control. While the results of interaction groups 1 and 2 showed significant increase in the lymphocyte account (5,148.67 and 5,541.67 cell/cu.mm.blood, respectively), as compared with positive control (1,148 cell/cu.mm.blood).

3.3 Monocyte

The MTX and distal water treatments were associated with a significant reduced count of monocytes (108.33 and 100 cell/cu.mm.blood) as compared with methanol extract treatment at the concentration (100 mg/ml) which associated with increase a monocyte count, but non-significant level was reached in the interaction treatment in both groups as compared to the negative and positive controls (Table 5).

3.4 Neutrophil

A treatment with MTX caused a significant reduction in neutrophil count (198.33 cell/cu.mm.blood) as compared to negative control (250 cell/cu.mm.blood).

The first dose of methanol extract decreased the count of neutrophils, while the second dose increased such count as compared to the corresponding negative and positive controls (Table 6), however, such differences did not attend a significant level. In the same way, the interaction treatment showed significant differences in the neutrophil account depending on the dose (123 and 266.67 cell/cu.mm.blood), as compared to the corresponding controls.

Table 3 Effect of *A. millefolium* methanolic extract and MTX on total count of leukocytes in albino male mice.

Groups	Mean ± stander error
A. millefolium 100 mg/mL	9,200.00 ± 611.01 A
A. millefolium 200 mg/mL	6,174.00 ± 230.94 C
Negative control (distal water)	7,500.00 ± 288.67 B
Positive control (MTX 40 mg/mL)	1,532.33 ± 581.18 E
Interaction Group 1 (MTX 40 mg/mL + *A. millefolium* 100 mg/mL)	5,333.33 ± 176.38 D
Interaction Group 2 (MTX 40 mg/mL + *A. millefolium* 200 mg/mL)	5,866.66 ± 185.59 D

Different letters in the same column: Significant difference ($P \leq 0.05$) between means.

Table 4 Effect of *A. millefolium* methanolic extract and MTX on lymphocyte cells in albino male mice.

Groups	Mean ± stander error
A. millefolium 100 mg/mL	8,904.00 ± 667.05 A
A. millefolium 200 mg/mL	5,800.00 ± 113.03 C
Negative control (distal water)	7,130.33 ± 260.63 B
Positive control (MTX 40 mg/mL)	1,148.00 ± 610.04 E
Interaction Group 1 (MTX 40 mg/ml+ *A. millefolium* 100 mg/mL)	5,148.67 ± 179.91 D
Interaction Group 2 (MTX 40 mg/ml+ *A. millefolium* 200 mg/mL)	5,541.67 ± 174.60 D

Different letters in the same column: Significant difference ($P \leq 0.05$) between means.

Table 5 Effect of *A. millefolium* methanolic extract and MTX on monocyte cells in albino male mice.

Groups	Mean ± stander error
A. millefolium 100 mg/mL	204.00 ± 63.79 A
A. millefolium 200 mg/mL	96.00 ± 18.15 B
Negative control (distal water)	100.00 ± 14.43 B
Positive control (MTX 40mg/mL)	108.33 ± 22.04 B
Interaction Group 1 (MTX 40 mg/Ml + *A. millefolium* 100 mg/mL)	61.67 ± 6.691 B
Interaction Group 2 (MTX 40 mg/mL + *A. millefolium* 200 mg/mL)	75.00 ± 14.43 B

Different letters in the same column: Significant difference ($P \leq 0.05$) between means.

Table 6 Effect of *A. millefolium* methanolic extract and MTX on neutrophil cells in albino male mice.

Groups	Mean ± stander error
A. millefolium 100 mg/mL	92.00 ± 6.11 B
A. millefolium 200 mg/mL	278.00 ± 39.72 A
Negative control (distal water)	250.00 ± 28.87 A
Positive control (MTX 40 mg/mL)	198.33 ± 22.05 B
Interaction Group 1 (MTX 40 mg/mL + *A. millefolium* 100 mg/mL)	123.00 ± 12.74 B
Interaction Group 2 (MTX 40 mg/mL + *A. millefolium* 200 mg/mL)	266.67 ± 44.18 A

Different letters in the same column: Significant difference ($P \leq 0.05$) between means.

4. Discussion

Assessment of hematological parameters can be used to explain hematological functions of a chemical compound or plant extracts in albino male mice [13]. Blood acts as a pathological reflector of the status of exposed animals to toxicants and other conditions or agents [14].

This study involved studying the methanol extract of *A. millefolium* on leucocyte blood cells on albino male mice. The results showed that methanol extract for this plant has high detoxification effect against the toxicity of MTX drug. Researchers were suggested that the drug generates DNA double strand breaks through its effect on the formed topoisomerase II cleavable complex, and such effect mimics the action of ionizing radiation. A radiation-induced DNA damage has been ascribed to the production of cytokines, in particular, interleukin and its induction of release have been correlated with DNA formulation [15]. Others have suggested that the drug is activated by the oxidative effects of the some enzyme, which is an abundant protein in neurophils and monocytes, and such enzyme oxidizes MTX to metabolites that can bind covalently to DNA and RNA [16]. According to study on the phytochemical constituents of *A. millefolium* showed that methanol extract of this plant contains Alkaloids (betonicine, stachydrine, trigonelline), Coumarins, Flavonoids (apigenin, luteolin, quercitin), Salicylic acid, Sesquiterpene lactones (achillin, achillicin), Polyacetylenes, Volatile oil with variable content (linalool, camphor, sabinene, chamazulene), Triterpenes, Tannins, and Sterols and plant acids. Many reports improved flavonoids, phenolics and aromatics are the most important pharmacological active constituents that are powerful anti-oxidants. For this reason, *A. millefolium* is considered a natural source of anti-oxidants, and the anti-oxidant activity of such extracts may play an important role in their anti-proliferative activities [17]. Flavonoids can significantly cause reduction of hydrogen peroxide which means scavengers of reactive oxygen species (ROS) in humans, flavonoid concentration in plasma and most tissues is too low to effectively reduce ROS. Instead, flavonoids may play key roles as signaling molecules in mammals, through their ability to interact with a wide range of protein kinases, including mitogen-activated protein kinases (MAPK), which supersede key steps of cell growth and differentiation [18]. The anti-oxidant activity of *A. millefolium* and its ability to sequester reactive oxygen species have been investigated by Ferda and his colleagues (2003), who studied the biological effects of different extracts, a correlation was observed between the anti-oxidant activity and chemical composition of its different fractions, with special emphasis on the presence of flavonoids and tannic acid derivatives. The study concluded that the components of *A. millefolium* act by different mechanisms sequestering reactive oxygen species. Additionally, several studies have confirmed that the pharmacological properties of *A. millefolium* are attributed mainly to the presence of favonoids as a result of their action against free radicals [19], and these polyphenols interfere not only with the propagation but also with the formation of free radicals both by chelating transition metals and by inhibiting enzymes involved in the initiation reaction [20].

Administration of methanol extract of *A. millefolium* led to significant increase in the levels of white blood cells that may have immune boosting properties. The increase in WBC count may have been due to enhancement in the rate of entry of leucocytes into the blood from the bone marrow. Granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor and interleukins (IL-2, IL-4 and IL-5) regulate the proliferation, generation and maturation of committed stem cells responsible for the production of WBCs. However, such increase in WBC counts may be due to over production of these haematopoietic regulatory elements by the stromal cells and macrophages in the bone marrow [21]. These effects may result from associated with the adjuvant activity of

some phytochemicals found in the extract. Alkaloids, tannins, phenolic compounds and flavonoids have been reported generally as immunostimulants [22].

5. Conclusions

The present study showed that *A. millefolium* leaf extract has a significant increase in total white blood cell and differential white blood cell counts in normal mice, after intraperetoial administration of the extract may promote the immune-stimulatory activities. These stimulant effects could be associated with the adjuvant activity of some phytochemicals found in the extracts hence which can be used in management of immune dependent disorders.

The present study, therefore, scientifically confirms and supports the traditional use of leaves of *A. millefolium* in enhancing detoxification properties on albino male mice.

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