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Research Article

Investigation of Anti-Inflammatory Properties, Phytochemical Constituents, Antioxidant, and Antimicrobial Potentials of the Whole Plant Ethanolic Extract of *Achillea santolinoides* subsp. *wilhelmsii* (K. Koch) Greuter of Balochistan

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Medicinal plants are rich source of phytochemical constitutes and can be used to treat many human diseases. Infectious diseases have always been a major source of concern. Globally, the medicinal plant *Achillea wilhelmsii* locally known as Bohe Madran is extensively dispersed and widely used as traditional medicine. The aim of this present work is to investigate phytochemical constituents and antimicrobial, antioxidant, and anti-inflammatory properties of the whole plant ethanolic extract of *Achillea santolinoides* subsp. *wilhelmsii* (WEEAW) from Balochistan region. The total phenolic content was 14.81 ± 0.18 mg GAE/g of the extract whereas the total flavonoid content was 12.27 ± 0.12 mg QE/g of the extract. The antioxidant ability of the extract was analyzed by DPPH (2,2-diphenyl-1-picryl-hydrazyl) scavenging assay and FRAP (ferric reducing antioxidant power) assay in terms of concentration having 50% inhibition (IC_{50}). Results showed that IC_{50} value for DPPH% inhibition was 0.367 ± 0.82 mg/mL while FRAP assay represented with IC_{50} value of 0.485 ± 1.26 mg/mL. In antileishmanial bioassay, the extract was analyzed against Leishmania major and showed good activity with IC_{50} value of 7.02 ± 0.83 mg/mL. Antibacterial assay revealed that *Staphylococcus aureus* was highly sensitive with the diameter of inhibition zone (21.61 ± 1.09 mm) followed by *Salmonella typhi* (17.32 ± 0.15 mm), *Pseudomonas aeruginosa* (16.41 ± 0.63 mm), and *Escherichia coli* (15.30 ± 1.17 mm) while *Klebsiella pneumoniae* showed minimum inhibition (14.13 ± 0.49 mm). Antifungal activity was tested against *Aspergillus flavus* with 89% of inhibition zone and 77% against *Mucor mucedo* and *Aspergillus niger* with 74% of inhibition zone. The anti-inflammatory assay was carried out by inhibiting protein denaturation, proteinase inhibitory activity, and heat-induced hemolysis. The IC_{50} value for protein denaturation was 6.67 ± 1.25 mg/mL, proteinase inhibitory with IC_{50} value of 4.12 ± 0.69 mg/mL, and heat-induced hemolysis assay with IC_{50} value 4.53 ± 0.82 mg/mL by comparing to the standard drug aspirin having IC_{50} value 1.85 ± 0.54 mg/mL. Results of the current work showed that whole plant ethanolic extract of *Achillea wilhelmsii* exhibited substantial anti-inflammatory action, thus can be utilized as a traditional treatment. Furthermore, overall finding of this research suggested that the antioxidant potential of the plant aids to prevent free radical damage and reduce the incidence of chronic disease. More research is needed to find out more active compounds present in the extract that are responsible for their pharmacological effects.

1. Introduction

Medicinal plants naturally produce a variety of phytochemical constitutes as secondary metabolites which can be used to treat many human diseases. Additionally, plant secondary metabolites have a great potential for producing novel medications and have been successfully employed to treat chronic and infectious diseases [1]. The World Health

Organization (WHO) estimates that 80 percent of the world's population still relies on indigenous medications for basic healthcare due to their ease of access [2]. These plants have significant antibacterial, antifungal, anti-inflammatory, antioxidant, anticancer, and antidiabetic properties and used for the treatment of various infections [3]. The natural antimicrobial agents present in medicinal plants have strong resistance to the usual antibiotics and drugs. As an alternative, these plants provide a traditional substitute to produce traditional drugs with low cost and easily available for local communities. In the future, such plants may play a larger role in disease control [4].

Achillea wilhelmsii is a perennial herb commonly known as Bohe Madran and Zawal in Brahui and Pashto languages. The plant belongs to Asteraceae or Compositae family, and the genus *Achillea* comprises over 120 species worldwide [5]. The plant is widely distributed in Asia particularly in Pakistan, Iran, and India [6]. The word *Achillea* was derived from the Greek word Achilles, which means "hero," and was mostly used to cure various diseases during battles [7]. The plant is best known for its pleasant fragrant aroma and traditionally used for treating a variety of health problems by local community [8]. *Achillea wilhelmsii* contains various chemical constituents including alkamides, alkaloids, carvacrol, linalool, α- and β-pinene, borneol, sesquiterpenoids (wilhemsin and wilhelmsolide), monoterpenoids, caryophyllene, rutin, thujene, 1,4-cineol, and camphor [6]. It has been shown that *Achillea* contains bitter aromatic substances having imperative effects on nervous system and neurological diseases such as epilepsy, neurasthenia, and seizures [9]. Many research studies documented that fractions, extracts, pure chemical compounds, and essential oils derived from *Achillea* species have a wide range of biological functions such as cytotoxic, antidiabetic, antispasmodic, antianxiety, anticancer, anti-inflammatory, analgesic, antibacterial, anticholinesterase activities [10, 11]. The plant decoction is used to treat children's motion, jaundice stomach pain, and fever, whereas green tea made from young shoots is used to treat stomach complications [12]. Inflammation is a complicated biological response of bodily tissues to adverse stimuli such as infections, damaged cells, or irritants and is characterized in vascular tissues by redness, warmth, swelling, and discomfort. Acute inflammation is a short-term process characterized by pain, redness, immobility, swellings, heat, and loss of body functions. Available anti-inflammatory medicines alleviate symptoms, suppress enzyme activities, and come with complicated negative side effects [2]. Consequently, using anti-inflammatory drugs with fewer side effects is also crucial.

The in vitro analysis of phytochemical constituents, antibacterial, antifungal, anti-inflammatory, and antileishmanial potentials of the ethanolic extracts of *Achillea wilhelmsii* variety available in Quetta Balochistan has not been reported. Therefore, the plant was evaluated for its antibacterial, antifungal, antioxidants, antileishmanial, and anti-inflammatory potentials against different microorganisms using an in vitro assay.

2. Materials and Method

2.1. Plant Collection and Sample Preparation. *Achillea wilhelmsii* plants were collected from Quetta region Balochistan with the area coordinates 30.623926 N, 67.323998 E. The plant was collected and identified by the Botanist Zareen Gul Department of Botany University of Balochistan Quetta. One part of the collected plant was dried preserved and kept at the Food Microbiology and Bioprocess Technology Laboratory Department of Microbiology University of Balochistan for reference. The other part of the plant was washed with distilled water and dried for a few weeks in the shade at room temperature with controlled humidity. Using an electronic grinder, the dried plants were crushed into fine powder and stored in desiccators for future investigation.

2.2. Extraction of Bioactive Compounds by Maceration. For extraction, 200 g of fine ground powder was extracted using 2 L of 70% ethanol as the solvent in a 1 : 10 ratio for 24 hours, as indicated by Gul et al. [2]. The treatment was performed in a dark environment to avoid light exposure. The flask was shaken at regular intervals. The ethanolic mixture was filtered using Whatman filter paper no. 1. The *Achillea santolinoides* subsp. *wilhelmsii* (K. Koch) Greuter whole plant ethanolic extract (WEEAW) obtained was dried in a rotary evaporator at 65°C and used for further research.

2.3. Phytochemical Analysis. The WEEAW was subjected to determine the presence of different phytochemical constituents (alkaloids, steroids, tannins, saponins, flavonoids, glycosides, terpenoids, quinones, coumarin, carbohydrates, and phenolic compounds) using standard procedures as described by Akbar et al. [13].

2.4. Estimation of Total Phenolic Content. The total phenolic content (TPC) of the WEEAW was determined using the Folin-Ciocalteu reagent technique using gallic acid as a standard, and the results were represented in milligrams of gallic acid equivalents (mg GAE/g) of sample dried weight [14]. In brief, 0.5 mL (1 mg/mL) dried crude plant extract was appropriately combined with newly made (2 mL) Folin-Ciocalteu reagent. After 5 minutes at room temperature, the mixture was neutralized with 2 mL of 10% Na_2CO_3 and incubated for 30 minutes. The absorbance at 750 nm was calculated using a T60 UV-Visible Spectrophotometer (PG, UK) and a blank of 95% ethanol.

2.5. Total Flavonoid Content. Total flavonoid contents (TFC) of the WEEAW were determined by aluminum chloride colorimetric assay as explained by Sadiq et al. [15]. Quercetin was employed as a control, and the results were represented in milligrams of quercetin equivalents per gram of the sample (mg QE/g). Simply, 0.5 mL of the extract (1 mg/mL) was combined with 95 percent ethanol and 0.5 mL ($NaNO_2$ 5%) solution. After 5 minutes (10% w/v, 0.1 mL) of $AlCl_3.6H_2O$, 0.5 mL of 1 M NaOH and 2 mL of deionized water were added and incubated at 25°C for 40 minutes. Absorbance was measured at 415 nm against a blank using a T60 UV-Visible Spectrophotometer (PG, UK). The measurements

were done using a standard curve and varied amounts of quercetin.

2.6. DPPH Radical Scavenging Activity. The antioxidant activity of WEEAW was assessed based on the radical scavenging effect on (2,2-diphenyl-1-picryl-hydrazyl) DPPH free radical. From the stock solution, several doses (1-0.0625 mg/mL) of the extract were prepared, and 50 μL of the extract was mixed with 5 mL of freshly prepared DPPH (40 ppm in ethanol) solution. The reaction mixture was mixed and incubated at room temperature in the dark for 30 minutes. Ascorbic acid was utilized as a control. The decrease in absorbance at 517 nm was used to detect DPPH decolorization. As a blank and control, ethanol and DPPH solutions without plant extract were utilized [15].

The following equation was used for % inhibition calculation:

$$\%\text{inhibition of DPPH} = \frac{\text{AC} - \text{AS}}{\text{AC}} \times 100. \quad (1)$$

Here, AC is the absorbance of control (DPPH), and AS is the extract absorbance.

The relationship curve was created by graphing the scavenging activity against various extract concentrations and was expressed in mg/mL. The results were presented as IC_{50} values (concentration of sample required to scavenge 50 percent of free radicals).

2.7. Ferric Reducing Antioxidant Power (FRAP) Assay. For the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)TPTZ] to the ferrous complex, the procedure from Benzie and Strain [16] was followed with little modifications. Suspension of FRAP was prepared by adding acetate buffer (0.3 M) pH 3.6, $FeCl_3{\cdot}6H_2O$ (0.02 M), and TPTZ (0.01 M) in HCl (0.04 M), respectively. In the dark, acetate buffer (25 mL), TPTZ solution (2.5 mL), and ferric chloride hexahydrate solution (2.5 mL) were mixed and incubated for 30 minutes at 37°C. Briefly, 0.5 mL of (WEEAW) 1 mg/mL was mixed with 2 mL of FRAP suspension followed by incubation at 37°C for 30 min in the dark, and absorbance was read at 595 nm, and % reduction was determined by the following equation:

$$\text{FRAP\%reduction} = \frac{\text{AC} - \text{AS}}{\text{AC}} \times 100, \quad (2)$$

where AC is the absorbance of control and AS is the absorbance of the extract.

2.8. Antileishmanial Assay. To accomplish antileishmanial assay of WEEAW, the *L. major* (promastigotes) were grown in culture medium Novy–Mac Neal–Nicolle (NNN) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) followed by incubation at 37°C for 72 h. The log phase of promastigotes at 1×10^6 cells/mL was used, and the assay was performed by means of a 96-well plate method described by Gul et al. [2]. Briefly, stock solution (1 mg/mL) of the test sample was prepared in DMSO. Twofold serial dilutions of the samples were carried out at different concentrations (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL), respectively. 10 μL of each dilution and 50 μL promastigotes log phase culture were dispensed to each well of 96-well plate. Glucantime (Meglumine antimonate) was employed as a reference medication and DMSO as negative control. The titer plate was incubated at 37°C for 72 h. Following the incubation period, 1 mL of DMSO was added to each well, and the percent mortality of the test and control medicines was evaluated using 20 L (5 mg/mL in phosphate buffer pH 7.2) of nitro blue tetrazolium (NBT) chloride solution. The absorbance at 630 nm was measured using a Microplate Reader (RT-6000), and the results were represented as a mean percent suppression in parasite population. The extract's IC_{50} values were computed using the linear regression approach, and the percent cell viability was obtained using the following formulas:

$$\%\text{cell viability} = \frac{A_{630}\text{of test sample}}{A_{630}\text{ of control}} \times 100,$$
$$\%\text{inhibition} = 100 - \%\text{viability}. \quad (3)$$

2.9. Antibacterial Activity. The antibacterial activity of WEEAW was determined by agar well diffusion method described by Gul et al. [2]. Freshly prepared Muller Hinton Agar (Oxoid UK) media were sterilized and inoculated with the 100 μL of target bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*) in different flasks and poured into sterilized plates. Wells were made through 6 mm sterilized cork borers in agar plates followed by adding 100 μL of the extract (1 mg/mL) and kept for incubation for 24 hr at 37°C. The antibiotic doxycycline was employed as a positive control, whereas dimethyl sulfoxide (DMSO) was used as a negative control. The diameter of the clean zone around the wells was measured in millimeters (mm) to obtain the results.

2.10. Antifungal Activity. Antifungal activities of WEEAW were determined by the method of Gul et al. [2]. Freshly produced Potato Dextrose Agar (Oxoid UK) was mixed with 1 mL of the extract and placed into Petri plates. After solidification, 6 mm diameter wells were created into agar by cork borer, and the actively growing test fungi (*Aspergillus flavus*, *Aspergillus niger*, and *Mucor mucedo*) were poured into various wells and incubated at 37°C for 72 h. The positive control contained media containing organisms without extract, while the negative control was merely media. Fluconazole, an antifungal drug, was employed as a reference. After five days of incubation, the results of the growth inhibition of the target fungal species were recorded. The growth inhibition of target fungus was measured in millimeters (mm) in comparison to the negative control. The following equation was used to calculate the final findings:

$$\%\text{inhibition} = \frac{100 - \text{linear growth in test(mm)}}{\text{linear growth in control(mm)}} \times 100. \quad (4)$$

2.11. Anti-Inflammatory Activity Determination

2.11.1. Inhibition of Albumin Denaturation. Anti-inflammatory effect was assessed using the inhibition of albumin denaturation assay, as described by Yesmin et al. [17]. The reaction suspension (5 mL) contained 1 mL of the extract (1 mg/mL), 3.8 mL of phosphate buffered saline (PBS, pH 6.4), and 0.2 mL of aqueous solution containing 1 percent bovine albumin. After incubation for 15 minutes at 37°C in a water bath, the reaction mixture was heated to 70°C for 5 min. After cooling, the absorbance at 660 nm was measured using UV-VIS Spectrophotometer (T60 UK). Acetylsalicylic acid was used as the standard medicine, and phosphate buffer solution was used as the control. The following formula was used to compute the % inhibition of protein denaturation:

$$\%\text{inhibition of protein denaturation} = 100 \times \frac{1 - \text{A2}}{\text{A1}}, \quad (5)$$

where $A1$ is the absorption of the control sample and $A2$ is the absorption of the test sample.

2.11.2. Proteinase Inhibitory Activity. Proteinase inhibitory activity of WEEAW was measured according to the method described by Gunathilake et al. [18] to examine the in vitro anti-inflammatory action. The reaction suspension (2 mL) was made up of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL of the extract. The mixture was incubated for 5 minutes at 37°C before adding 1 mL of 0.8 percent (*w*/*v*) casein and incubating for another 20 minutes before adding 2 mL of 70% perchloric acid and centrifuged. At 210 nm, the absorbance of the supernatant was measured using a phosphate buffer solution as a control and acetylsalicylic acid as a reference medication for the experiment. The percentage inhibition was calculated by using the following formula:

$$\%\text{proteinase inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}. \quad (6)$$

2.12. Heat-Induced Hemolysis Assay. Erythrocyte suspension was prepared following Gul et al. [2] with some modifications. A healthy individual's whole blood was obtained. To remove supernatants, the blood was centrifuged at 2000 × g for 5 min. The mixture was rinsed with an equivalent volume of normal saline (0.9 percent NaCl) and centrifuged for 5 minutes at 2000 × g. The procedure was performed three times, and the blood volume was calculated and reconstituted as a 10% (*v*/*v*) suspension with an isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). The buffer solution used (g/L) contained $NaH_2PO_4.2H_2O$ (0.2) g, Na_2HPO_4 (1.15) g, and NaCl (9.0) g.

Heat-induced hemolysis assay was carried out as described by Yesmin et al. [17] with some modifications. In brief, 0.06 mL of blood cell suspension and 0.06 mL of the extract were combined with 2.93 mL of phosphate buffer (pH 7.4) before being incubated in a water bath at 54°C for 20 minutes. The mixture was centrifuged again (2000 × g for 3 min), and the absorbance of the supernatant was measured at 540 nm using a microplate reader (T60 UV VIS Spectrophotometer). For the experiment, a phosphate buffer solution was utilized as a control, and acetylsalicylic acid was employed as a standard medication. The following equation was used to compute the % inhibition of hemolysis:

$$\text{Percentage inhibition of hemolysis} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}, \quad (7)$$

where $A1$ is the absorption of the control and $A2$ is the absorption of test sample.

2.13. Statistical Analysis. MS Excel 2010 software was used to compute the magnitude of the means, standard curve, and standard deviations. The findings are presented as the mean SD of three replicates. Linear regression method was used to measure the inhibitory concentrations (IC_{50}) value.

3. Results

3.1. Phytochemical Analysis. The phytochemical evaluation of WEEAW confirmed the presence of alkaloids, flavonoids, terpenoids, anthraquinones, saponins, tannins, coumarin, steroids, carbohydrates, and phlobatannins. However, cardiac glycoside and quinones were absent (Table 1). These investigations also revealed that *Achillea wilhelmsii* contains a diverse variety of phytochemical compounds.

3.2. Phenolic Contents. The TPCs of WEEAW were evaluated by using Folin-Ciocalteu method while gallic acid was used as a reference for standard. The TPCs of the extract were recorded 14.81 ± 0.18 mg GAE/g (Table 2).

3.3. Flavonoid Contents. The total flavonoid content was determined, and the results were computed using the calibration curve and represented as mg quercetin equivalents (QE) per gram of dry weight sample. Total flavonoid contents in extract were 12.27 ± 0.12 mg QE/g as presented in Table 2.

3.4. Quantitative Assay for DPPH Free Radical Scavenging Activity. In the presence of DPPH stable radical, the WEEAW hydrogen donating ability was determined, and its reducing potential was calculated based on their concentration indicating 50% inhibition (concentration of the extract needed to scavenge 50 percent DPPH free radicals). The results were obtained using a linear regression equation formed by the concentration of the extract versus their percent scavenging ability. According to the findings, the extract's radical scavenging activity increased with increasing concentration (Table 3 and Figure 1(a)). In current study, the DPPH radical scavenging activity was confirmed with IC_{50} (0.367 ± 0.82 mg/mL) in comparison with the standard ascorbic acid having IC_{50} (0.251 ± 0.91 mg/mL) as presented in Table 4.

Table 1: Phytochemical constituents of the whole plant ethanolic extract of *Achillea wilhelmsii*.

S. no	Phytochemical test	Results
1	Alkaloids	+ve
2	Cardiac glycosides	-ve
3	Tannins	+ve
4	Steroids	+ve
5	Terpenoids	+ve
6	Flavonoids	+ve
7	Saponins	+ve
8	Coumarin	+ve
9	Carbohydrates	+ve
10	Quinones	-ve
11	Anthraquinones	+ve
12	Phlobatannins	+ve

Note: +ve = present; -ve = absent.

Table 2: Total phenolic contents and total flavonoid contents of the whole plant ethanolic extract of *Achillea wilhelmsii*.

Sample	Total phenolic (mg GAE/g) ± SD	Total flavonoid (mg QE/g) ± SD
WEEAW	14.81 ± 0.18	12.27 ± 0.12

Note: results are expressed as mean ± SD for three readings.

Table 3: DPPH % inhibitions, FRAP % reduction, and antileishmanial assay of the whole plant ethanolic extract of *Achillea wilhelmsii* at different concentrations.

Concentrations in (mg/mL)	DPPH % inhibition assay	FRAP % reduction assay	Antileishmanial assay
1	82.21 ± 1.52	88.42 ± 0.81	59.23 ± 0.67
0.5	68.33 ± 1.21	70.15 ± 1.01	52.08 ± 0.18
0.25	44.26 ± 0.96	51.23 ± 0.54	45.54 ± 0.26
0.125	37.17 ± 1.03	43.08 ± 0.76	37.19 ± 0.20
0.0625	23.09 ± 1.25	21.81 ± 1.13	29.24 ± 0.62

3.5. Ferric Reducing Antioxidant Power Assay. WEEAW's antioxidant capacity was assessed using the ferric reducing antioxidant power (FRAP) assay. The results were expressed as the concentration providing 50% inhibition (IC_{50}), which is the concentration of the extract required to decrease Fe^{3+} to Fe^{2+}. The linear regression equation formed by the concentrations of the extracts versus their percent reduction ability yielded the findings. According to the obtained result, the extract has FRAP % reduction activity with IC_{50} (0.485 ± 1.26 mg/mL) as presented in Table 4. The higher IC_{50} value indicates lower antioxidant potential and same for ferric reducing activity. In comparison with the standard having IC_{50} value (0.314 ± 0.97 mg/mL), WEEAW showed relatively lower antioxidant potential. The current studies demonstrated that the extract's radical scavenging activity increased with increasing concentration, as mentioned in Table 3 and Figure 1(b).

3.6. Antileishmanial Assay. Antileishmanial assay was used to investigate the hidden potential of WEEAW against promastigotes (*Leishmania major*). Results have shown that different concentrations (1, 0.5, 0.25, and 0.125 mg/mL) of the extract exhibited a significant antileishmanial activity with substantial % inhibition having IC_{50} value (7.02 ± 0.83 mg/mL) compared with standard drug Glucantime IC_{50} (4.32 ± 0.63 mg/mL) as shown in Table 4. The % inhibition decreased with decrease in concentration as presented in Table 3 and Figure 2.

3.7. Antibacterial Activity. Antibacterial assay of WEEAW was evaluated against different bacterial strains. Results were presented in diameter of the inhibition zones (Table 5). Among Gram-negative and Gram-positive bacteria, *Staphylococcus aureus* was observed highly sensitive with the diameter of inhibition zone (21.61 ± 1.09 mm) followed by *Salmonella typhi* (17.32 ± 0.15 mm), *Pseudomonas aeruginosa* (16.41 ± 0.63 mm), and *Escherichia coli* (15.30 ± 1.17 mm) diameter of the inhibition zones, respectively. The extract was found active against all selected bacteria except Gram-negative *Klebsiella pneumoniae* that showed minimum inhibition (14.13 ± 0.49 mm).

3.8. Antifungal Activity. The percentage of inhibition zone was used to assess WEEAW's antifungal activity against three filamentous fungi (*A. flavus*, *A. niger*, and *M. mucedo*). When compared to the antifungal medication fluconazole, the extract demonstrated substantial antifungal action against fungus strains. The extract exhibited substantial antifungal action against Aspergillus flavus (89% inhibition) and *Mucor mucedo* (77% inhibition) but had a very minor effect on the growth of *A. niger* (74% inhibition). Fluconazole, an antifungal medication, inhibited *M. mucedo* by 98%, *A. niger* by 95 percent, and *A. flavus* by 94% (Figure 3 and Table 6).

3.9. In Vitro Anti-Inflammatory Assay. To examine the anti-inflammatory properties, WEEAW was subjected to preliminary analysis using known procedures. The results of in vitro anti-inflammatory activity for inhibition of protein denaturation, proteinase inhibition, and heat-induced hemolysis were presented in Table 7. Results of the current work revealed the effectiveness of the extract. Comparing results with reference drug aspirin IC_{50} value (1.85 ± 0.54 mg/mL), maximum % inhibition of the extract was observed for proteinase inhibition with lowest IC_{50} value (4.12 ± 0.69 mg/mL) followed by heat-induced hemolysis (4.53 ± 0.82 mg/mL). Minimum % inhibition was seen for inhibition of protein denaturation with highest IC_{50} value (6.67 ± 1.25 mg/mL), respectively. The highest activity was observed in concentration of (1 mg/mL), and the viability increased with decreased in concentration presented in Figure 4.

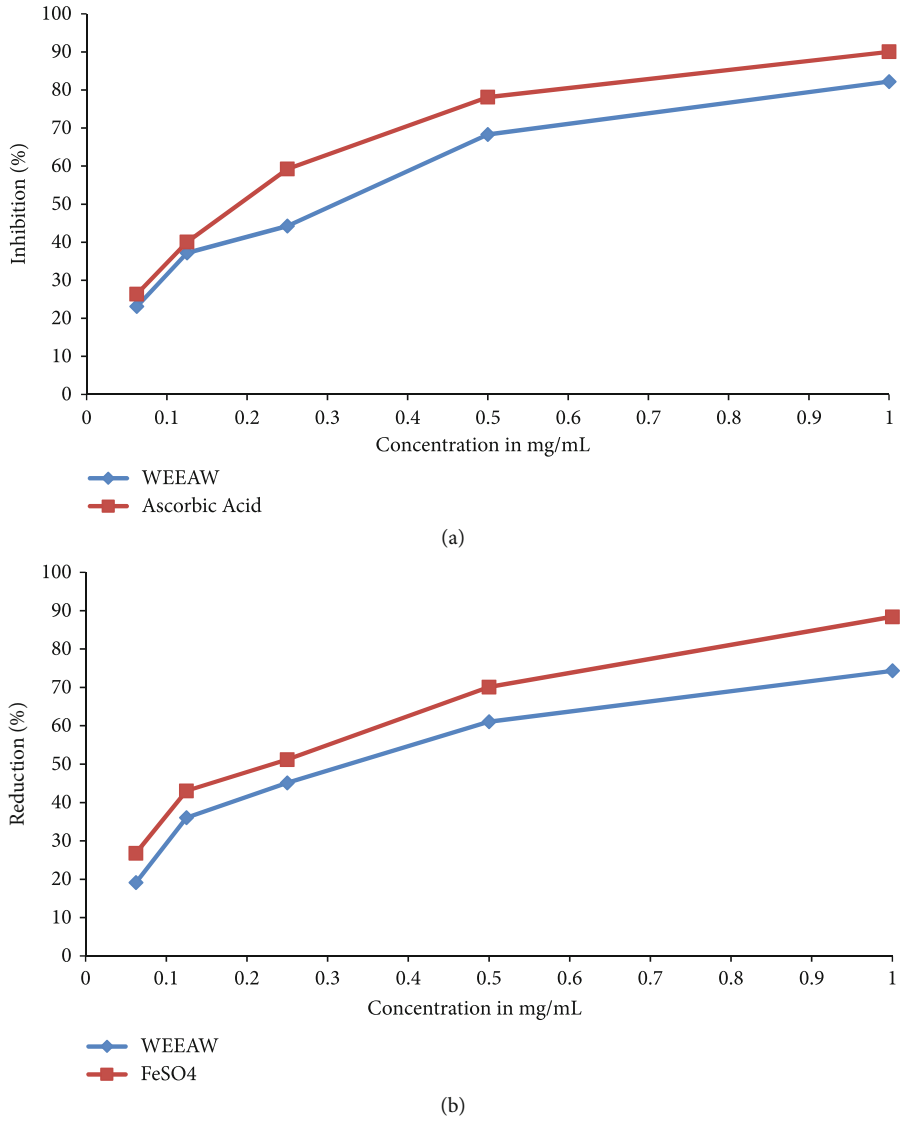

Figure 1: (a) Free radical scavenging activity (DPPH). (b) Ferrous reducing capacity (FRAP) of the whole plant ethanolic extract of *Achillea wilhelmsii*.

Table 4: Estimated IC_{50} values of the whole plant ethanolic extract of *Achillea wilhelmsii* and standards.

Assay	Samples	IC_{50} value (mg/mL)
DPPH	WEEAW	0.367 ± 0.82
	Ascorbic acid	0.251 ± 0.91
FRAP	WEEAW	0.485 ± 1.26
	$FeSO_4$	0.314 ± 0.97
Antileishmanial	WEEAW	7.02 ± 0.83
	Glucantime	4.32 ± 0.63

Note: ascorbic acid, $FeSO_4$, and Glucantime are used as a standards for the studies.

4. Discussion

Plants with high phytochemical constituents are beneficial to health because of their ability to reduce the risk of various ailments such as heart diseases, diabetes, and certain cancers [19]. The present study was conducted to evaluate the phytochemical constituents and antimicrobial and antioxidant activities of the whole plant ethanolic extract of *Achillea wilhelmsii* (WEEAW) from different regions of Balochistan. Alkaloids, tannins, phlobatannins, anthraquinones, saponins, flavonoids, phenolics, carbohydrates, coumarin, steroids, and terpenoids were discovered. All the chemicals found are physiologically active, with antibacterial, antifungal, antiviral, antiparasitic, and antioxidant properties. Alkaloids are anti-inflammatory and analgesic substances that serve to boost the immune system, relieve pain, and are used to treat snakebite, skin disorders, and asthma. Alkaloids provided the basic structure for the development of numerous

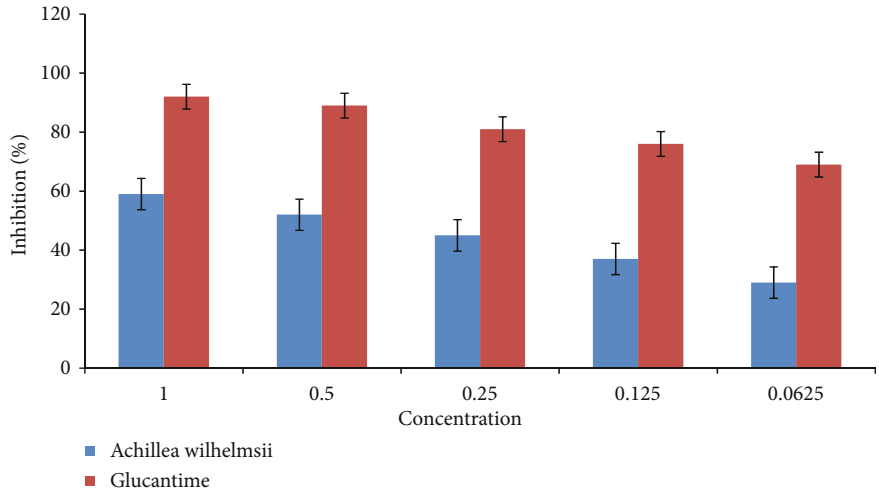

Figure 2: Antileishmanial activities of the whole plant ethanolic extract of *Achillea wilhelmsii* against promastigotes (*Leishmania major*). Bars represent the standard deviation of the mean.

Table 5: Antibacterial activity of the whole plant ethanolic extract of *Achillea wilhelmsii* with inhibitory zones.

Samples	Diameter of zone of inhibition against target pathogens in millimeters (mm)				
	E. coli	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*	*S. Typhi*
WEEAW	15.30 ± 1.17	14.13 ± 0.49	16.41 ± 0.63	21.61 ± 1.09	17.32 ± 0.15
Doxycycline	20.61 ± 2.85	19.37 ± 2.48	12.62 ± 2.67	21.12 ± 1.41	14.60 ± 2.85
DMSO	0	0	0	0	0

Note: *E. coli* = *Escherichia coli*; *K. pneumoniae* = *Klebsiella pneumoniae*; *P. aeruginosa* = *Pseudomonas aeruginosa*; *S. aureus* = *Staphylococcus aureus*; *S. typhi* = *Salmonella typhi*.

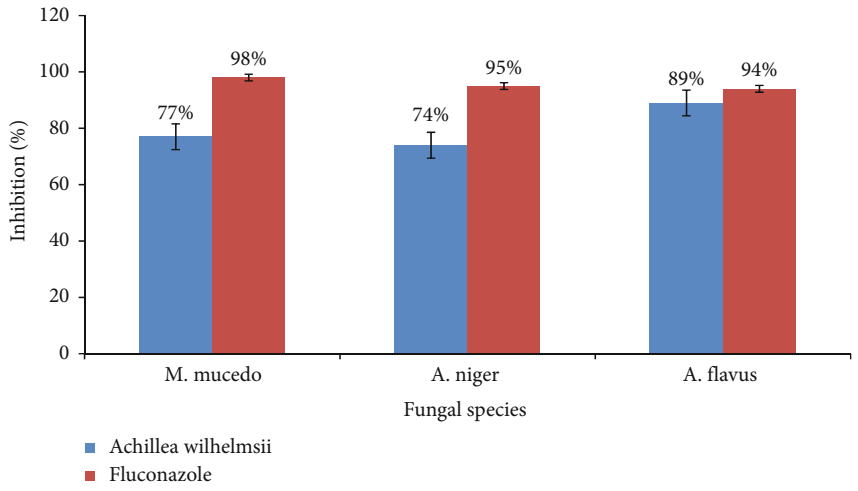

Figure 3: Antifungal activity of the whole plant ethanolic extract of *Achillea wilhelmsii* against fungal species *Mucor mucedo*, *Aspergillus niger*, and *Aspergillus flavus*. Bars represent the standard deviation of the mean.

antibiotics with a diverse actions [20]. Flavonoids have significant physiological impacts, including the ability to alter how the body responds to viral infection, allergies, and carcinogens. Literature also revealed the presence of several subgroups of terpenes in *Achillea* species [21]. Tannins also have antitumor and antidefensive activities, thus have ability to lower the blood pressure, speed up the blood coagulation, and lower the amount of lipid in serum [22]. Saponins have medicinal importance as it exhibits antioxidant, antimicrobial, antidiabetic, anticancer, anti-inflammatory, antispasmodic, and analgesic properties; hence, their presence in plant extract is a solid sign of its therapeutic usefulness [23]. An extensive range of secondary metabolites such as steroids, flavonoids, tannins, alkaloids, and terpenoids were

Table 6: Antifungal activity of the whole plant ethanolic extract of *Achillea wilhelmsii* against three fungal species.

ID	*M. mucedo*	*A. niger*	*A. flavus*
	Growth inhibition in percent		
WEEAW	77	74	89
Fluconazole	98	95	94

Note: percentage of inhibition: 0-39 = low; 40-59 = moderate; 60-69 = good; above 70 = significant activity.

found in *Achillea* species that has antimicrobial, antitumor, anti-inflammatory, antidiabetic, and antiradical potentials [24]. Current results of phytochemicals are in agreement with Serino et al. [6]. These findings imply the bioactive potential of *Achillea wilhelmsii*.

The phenolic content has been shown to have antibacterial, antioxidant, anti-inflammatory, and antidepressant properties. These compounds are deemed as active component of plants comprising good reducing potential due to which they act as good antioxidants. According to Raudone et al. [25], phenolic and flavonoid contents present in *Achillea wilhelmsii* were among particularly important group of metabolites because they contribute to substantial biological activity and may be related to antioxidant potential. In current study, the total phenolic contents of WEEAW were 14.81 ± 0.18 mg GAE/g. Previously, Fathi et al. [26] reported a higher TPC of *Achillea wilhelmsii* value 37.4 ± 0.3 mg GAE/g of the extract. According to the reported data of Jafari et al. [27], the total phenolic contents of *Achillea wilhelmsii* extract were 55.07 ± 0.295 mg GAE/g dry extract. Bashi et al. [28] documented that total phenolic contents of *Achillea wilhelmsii* methanolic extract value 17.18–59.61 mg GAE/g that were in agreement with the present findings. Recently, Şabanoğlu et al. [29] reported 119.4 ± 1.4 mg GAE/g extract of flower, 155.5 ± 8.7 mg GAE/g extract of leaves, and 136.4 ± 8.8 mg GAE/g extract for roots, respectively. The results of phenolic content were also in agreement with Asariha et al. [5]. Other study also revealed 104.66 mg GA/g of total phenolic contents for *Achillea santolina* by Yazdanparast et al. [30]. According to these findings, *Achillea* species appear to be a high source of phenolic acids and can be considered an auspicious natural antioxidant source [31]. Nevertheless, it has been illustrious that phenolic content assessed by spectrophotometry can be influenced by a variety of factors that comprise method of extraction, sampling treatment before processing, climatic condition, soil structure, and even plant age [32]. According to Sevindik et al. [33], *Achillea millefolium* petroleum ether extract resulted 85.72 ± 0.067 and methanolic extract with $77.78 \pm 0.145\,\mu$g GAE/mg extract. According to the findings of Gharibi et al. [34], the total phenolic contents varied from 15.55 mg in *Achillea aucheri* to 60.65 mg tannic acid/g of dry product in *Achillea pachycephala*.

Flavonoid contents are known as good antioxidants having strong reducing potentials. These components also comprise antimicrobial, anti-inflammatory, anticancer, and antidepressant potentials [35]. Among the metabolite classes found in Achillea species, flavonoids are the most abundant natural compounds in extracts. They can be found as aglycones and glycosides. Jafari et al. [27] documented higher total flavonoid contents of *Achillea wilhelmsii* which were 39.14 ± 0.100 mg rutin equivalent/g dry extract as compared to present study. Additionally, lower flavonoid content 2.5 ± 0.1 mg quercetin equivalent/g of the extract was also reported by Fathi et al. [26]. Another study, conducted in Iran, illustrates that the total flavonoid contents in *Achillea wilhelmsii* range from 7.79 to 12.61 mg catechin equivalent/g sample in maceration and ultrasound-assisted extraction [28] and stated that *Achillea wilhelmsii* has a lower total flavonoid content than *Achillea biebersteinii*. Several research studies have also shown that increasing flavonoids in the diet reduces the risk of a variety of human diseases [5].

In the present study, WEEAW was seen to have greatest antioxidant activity with IC_{50} value of 0.367 ± 0.82 mg/mL compared with ascorbic acid as standard. Results are in agreement with available data of Şabanoğlu et al. [29] with $IC_{50}1.500 \pm 0.024$, 0.812 ± 0.013, and 0.991 ± 0.017 mg/mL for flower, leaf, and root, respectively. Recently, Asariha et al. [5] reported that *Achillea wilhelmsii* GNPs and leaf infusion showed 58 and 68% of the scavenging effect at a concentration of 300 μg/mL, respectively. Previously, Fathi et al. [26] illustrated the DPPH radical scavenging activity of *Achillea wilhelmsii* with $IC_{50}58.9 \pm 2.7\,\mu$g/mL, and IC_{50} values for ascorbic acid, quercetin, and BHA were 3.7 ± 0.1, 3.9 ± 0.2, and $29.3 \pm 5.9\,\mu$g/mL, respectively. Another study reported by Alfatemi et al. [36] showed the antioxidant activity of essential oil of *Achillea wilhelmsii* with EC_{50} of 0.01 mg/mL, and scavenging activity EC_{50} was 0.58 mg/mL. Previous researchers documented that the antioxidant activities of essential oils *Achillea* can be attributed to their phenolic contents [37]. Present results are also in accordance with Bashi et al. [28]. Furthermore, a research study conducted in Iran revealed that IC_{50} of *Achillea wilhelmsii* was $154.5 \pm 1.01\,\mu$g/mL where the IC_{50} of BHT (standard) was calculated as $33.5 \pm 0.16\,\mu$g/mL. Hence, these results showed that antioxidant capacity of *Achillea wilhelmsii* was 0.21 time more than BHT [27]. In addition, Gharibi et al. [34] also reported the antioxidant activity of three Iranian endemic *Achillea* species of *Achillea aucheri*, *Achillea kellalensis*, and *Achillea pachycephala* and documented the antioxidant activity by DPPH assay with IC_{50} (248–844 μg/mL) and stated that the variation for IC_{50} among species might be attributed to differences in polyphenolic compounds. Furthermore, the methods of extraction and time can highly affect the results of DPPH assay. Similarly, Barış et al. [31] reported the antiradical activities of *Achillea biebersteinii*, *Achillea aleppica*, and *Achillea aleppica* subsp. *zederbaueri* ethanol extract through DPPH scavenging assay were 85%, 81%, and 73% with IC_{50} values of 33, 33, and 32 μg/mL, respectively. Likewise, Sevindik et al. [33] stated that *Achillea millefolium* ethyl acetate extract showed 55.1 ± 1.168 and methanolic extract displayed 23.7% inhibition in DPPH radical at 40 μg/mL.

The reducing capacity of a sample identifies a considerable potential antioxidant activity. Hence, the FRAP assay is used to examine the reduction of ferric iron (Fe^{3+}) into ferrous iron (Fe^{2+}). The results of this study displayed that WEEAW possessed adequate antioxidant power with IC_{50}

Table 7: IC_{50} (mg/mL) of in vitro anti-inflammatory activity whole plant ethanolic extract of *Achillea wilhelmsii* (WEEAW).

Test samples	Conc. (mg/mL)	Inhibition of protein denaturation IC_{50} (mg/mL)	Proteinase inhibition IC_{50} (mg/mL)	Heat induced hemolysis IC_{50} (mg/mL)
WEEAW	1 0.5 0.25 0.125 0.0625	6.67 ± 1.25	4.12 ± 0.69	4.53 ± 0.82
Aspirin	1 0.5 0.25 0.125 0.0625	1.85 ± 0.54	1.85 ± 0.54	1.85 ± 0.54

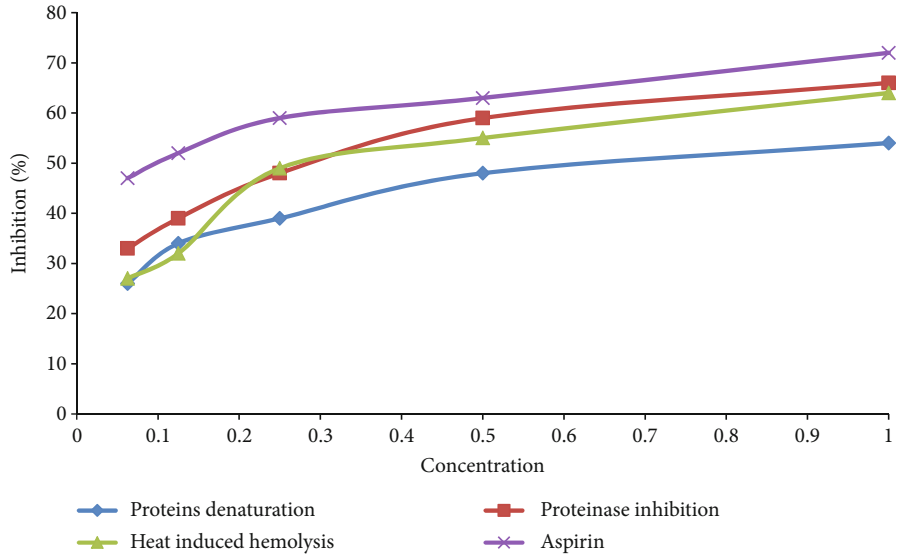

Figure 4: Anti-inflammatory activity of the whole plant ethanolic extract of *Achillea wilhelmsii*.

value of 0.485 ± 1.26 mg/mL. FRAP reducing power of *Achillea fragmentisma* methanol extract was recently studied. The results showed that plants gathered from two separate habitats had significantly higher reduction capacity in concentrations of 100 and 1000 μg/mL, with estimated IC50 values of 0.23 ± 0.02 g/L and 1.91 ± 0.05 g/L, respectively [38]. Likewise, Zengin et al. [39] demonstrated that methanolic extract of *A. biebersteinii* exhibited highest reducing power (196.12 mg TE/g extract). The methanolic extract of *Achillea coarctata* showed the moderate antioxidant activity with 1.74 mM/L FRAP value at 2 mg/mL compared with ascorbic acid which was 4.52 mm/L [40]. The higher the total phenolic and flavonoid contents, the higher will be the antioxidant potential. These ingredients are known to have antioxidant properties by either breaking the free radical chain or donating a hydrogen atom [29]. Alsohaili and Sulaiman [41] conducted a research study on essential oil of *Achillea tomentosa* and reported that the essential oil possessed lower ferric reducing power (EC 54.69 ± 4.69 μg/mL) compared to trolox (10.87 ± 3.34 μg/mL). Venditti et al. [42] analyzed *Achillea ligustica* All. composition and found that the flower and aerial parts contained secondary metabolites mainly sesquiterpenoids and flavonoid glucuronides. The crude extract obtained from aerial parts of *Achillea tenorii* was reported to be effective in α-glucosidase inhibition and antioxidant potential attributed to the presence of phenolic compounds [43]. The methanolic leave extract of *Achillea fragrantissima* was reported with antimicrobial and anticancer potential against various cancer cell lines [44].

Antileishmanial assay was used to investigate the hidden potential of ethanolic extract of *Achillea wilhelmsii* against *L. major*. The results confirmed that WEEAW exhibited a significant antileishmanial activity at different concentrations. Numerous antileishmanial investigations have already been conducted on the essential oil of *Achillea* species, including *Achillea wilhelmsii*. A result of a study conducted in Balochistan [8] revealed that whole plant n-hexane fraction

of *Achillea wilhelmsii* (WHFAW) has significant antileishmanial potential. The inhibitory concentration (IC_{50}) of the whole plant against *L. major* was found to be $58.27 \pm 0.52\,\mu g/mL$. According to Darwish et al. [45], *Achillea* plant with concentration $100\,\mu M$ showed antileishmanial action with $97.25\% \pm 1.63\%$ inhibition and stated that various phytochemicals present in *Achillea* genus have antileishmanial properties. Moreover, Al-Sokari et al. [46] investigated antileishmanial action of 16 different medicinal plant including *Achillea biebersteinii*, against *L. amazonensis* where *Achillea biebersteinii* flowers displayed the most promising activity with inhibition% value of ($IC_{50} = 26.9 \pm 2.9\,\mu g/mL$). Countless research studies identified that plant containing chemical constituents naturally such as alkamides identified from *Achillea* species was shown to have antileishmanial properties [46].

Antibacterial assay of WEEAW was evaluated, and the results were presented in diameter of the inhibition zones (Table 5). Among Gram-negative and Gram-positive bacteria, *Staphylococcus aureus* was observed highly sensitive with the diameter of inhibition zone (21.61 ± 1.09 mm) followed by *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Escherichia coli*, with the diameter of inhibition zone of 17.32 ± 0.15, 16.41 ± 0.63, and 15.30 ± 1.17 mm), respectively. While *Klebsiella pneumoniae* showed less sensitivity with the diameter of inhibition zone of (14.13 ± 0.49 mm). Previously, Amjad et al. [47] examine antibacterial activity of *Achillea wilhelmsii* against four pathogenic bacteria. The results revealed that the methanolic extract of *Achillea wilhelmsii* was the least efficient against *Pseudomonas aeruginosa* compared with other bacterial pathogens. Due to the differences in cell wall structure, Gram-positive bacteria are more sensitive than Gram-negative bacteria because their outer cell membrane acts as a barrier to different components, including antibiotics [48]. Some previous research studies found that *Achillea lingulata*, *Achillea millefolium*, and *Achillea clavennae* showed lower sensitivity to bacterial pathogens such as *S. enteritidis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* [33]. The results of the previous studies revealed that the essential oils of *Achillea wilhelmsii* are rich in monoterpenes, such as borneol, myrtenol, camphor, and 1,8-cineole, and these constituents have potent antibacterial effects [37]. Additionally, this plant naturally contains phenolic components, sesquiterpenes, lactones, and flavonoids having antibacterial properties [28]. It has been recently reported that *Achillea fragmentisma* methanol extract demonstrated good antibacterial activity against Gram-positive clinical isolate (*Bacillus cereus*, *Staphylococcus epidermidis*, and *Staphylococcus aureus*), with antibacterial activity ranging from high to no activity (between 14.5 ± 0.5 and 6.0 ± 0.0 mm zone of inhibition), but no effect on Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* [38].

Antifungal activity revealed that WEEAW exhibits potent antifungal activity against fungal strains. In the present study, *Aspergillus flavus* was observed highly sensitive with the percentage zone of inhibition (89%) followed by *Mucor mucedo* and *Aspergillus niger* with the percentage zone of inhibition 77% and 74% comparison with standard antifungal drug fluconazole with 98% zone of inhibition. Previously, Amjad et al. [49] reported that the methanolic extract of *Achillea wilhelmsii* C. Koch flowers has excellent antifungal activity against 20 Candida albicans strains. In contrast to the current work, Tajehmiri et al. [50] found that three fungal species *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus* were susceptible to *A. wilhelmsii* extract. Antimicrobial activity of *Achillea wilhelmsii* extract was also investigated by Bashi et al. [28], and their findings showed no obvious inhibitory effect on *Aspergillus niger*. A research work conducted in Iran investigated the antifungal activities of essential oil of *Achillea biebersteinii* against five pathogenic fungi. The results illustrated the strong antifungal activities against all tested fungal species [51]. Thus, according to the preceding research, the presence of saponins, tannins, alkaloids, terpenes, carotenoids, and flavonoids caused antifungal potentials [28].

The plant extract was subjected to determine its in vitro anti-inflammatory capabilities by using known procedures as inhibition of protein denaturation, proteinase inhibition, and heat-induced hemolysis. Protein denaturation occurs in inflammatory disorders such as rheumatoid arthritis and diabetes, as well as in cancer, and is one of the main causes of inflammatory diseases. Inflammatory disorders might be minimized by preventing protein denaturation. Anti-inflammatory medications could benefit from chemical compounds that prevent protein denaturation. In arthritic condition, proteinase plays a crucial role. A serine proteinase is copiously found in the lysosomal granules of neutrophils. Previous literature documented that during inflammatory reactions, leukocyte proteinase plays a significant role in the development of tissue damage; hence, proteinase inhibitors provide significant protection. There have been a few studies on anti-inflammatory activities of *Achillea* species. However, literature demonstrated that *Achillea* is a well-known genus to have anti-inflammatory potential as it comprises phenolic, flavonoids, tannins, saponins, terpenoids, sesquiterpenes, diterpenes, lignans, essential oil, and rarely triterpenes [6]. Additionally, alkamides were also well known for their anti-inflammatory properties [52]. Similarly, sesquiterpenes and lactones prevent DNA binding of transcription factors which control evolution of inflammation more effectively [53]. Recently, Honari et al. [54] reported the strong anti-inflammatory effect of *Achillea wilhelmsii* from Iran. Current results were also supported by the previous documented data of in vivo anti-inflammatory activity of five *Achillea* species [52].

5. Conclusion

The findings of the present study illustrated that whole plant ethanolic extract of *Achillea wilhelmsii* (WEEAW) exhibited significant antioxidant, antibacterial, antileishmanial and anti-inflammatory activities. The outcome of these findings suggests that the extract has significant medicinal benefits and possesses promising biological potentials. However, more research studies are needed to explore the bioactive molecules responsible for their efficiency.

Data Availability

Most of the data is part of the manuscript, and the remaining data will be made available on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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