International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2018; 6(1): xx-xx
Received: 09-01-2018
Accepted: 11-02-2018

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Antiradical, antimicrobial and cytotoxic activity evaluations of *Alchemilla mollis* (Buser) Rothm

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Abstract
Alchemilla mollis (Buser) Rothm. (Rosaceae) that grows in Turkey was examined for its different biological activities. The chemical composition of 50% methanol, water and deodorized water extract were determined spectrophotometrically (total phenol, total flavonoid and total flavonols). The antiradical activity was determined by DPPH• (1,1-diphenyl- 2-picrylhydrazyl) and $ABTS^{+\bullet}$ (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity assays. Antimicrobial activity was investigated by Agar Dilution Method. Finally the cytotoxic effect was evaluated on MCF 7 (human breast adenocarcinoma cell line) cell line with using Sulforhodamine B (SRB) method. The deodorized water extract showed higher antiradical activity in DPPH• method whereas water extract in $ABTS^{+\bullet}$ method. Water extract and deodorized water extract inhibited the viability of the MCF 7 cells by 70% at a concentration of 125.0 µg/mL. These results show that *A. mollis* may be a source of antiradical, antimicrobial and cytotoxic agent to support the medical use.

Keywords: *Alchemilla mollis*, Rosaceae, Antiradical activity, Antimicrobial activity, Cytotoxic activity

1. Introduction
Alchemilla is a genus of herbaceous perennial plants in the Rosaceae, with the common name Lady's Mantle [1]. There are about 300 species, the majority native to Europe and Asia, with a few species native to the mountains of Africa, North America and South America [2]. *Alchemilla mollis* (Buser) Rothm. is also known as Lady's Mantle and native to southern Europe and grown throughout the world as an ornamental garden plant. It grows up to 1m tall, with large and lobed leaves. Its greenish yellow flowers are arranged in large inflorescence and held in dense clusters above the foliage [3]. The word *Alchemilla* is a derivative of the Arab word Alkemelych, which means alchemy, and was named for the plant's magical healing powers [4]. In folk medicine, lady's mantle was also used to soothe infections of the mucous membranes of mouth and throat [5, 6]. Aerial part of the plant which is used medicinally, traditionally used for skin irritations, wounds, female conditions such as menorrhagia, menopause and painful periods. Lady's mantle was also used for treating blood sugar control diseases, although no evidence exists to support its usefulness [7].
Alchemillae herba is registered in European Pharmacopoeia 6.0 as a medicinal plant and it has been claimed to exhibit a variety of pharmacodynamics activities [8]. The tannins in *Alchemilla* species are responsible for the pharmacological activities such as antioxidant and antimicrobial [9]. *A. vulgaris* contains tannins composed of some gallic and mostly ellagic acid, flavonoids; the most abundant being quercetin, along with others, including luteolin and proanthocyanidins [5]. Therefore the results indicate that *A. vulgaris* seems to be candidate as a natural additive in food; cosmetic and pharmaceutical industries as its extracts possess strong antioxidant and protective effects. *A. vulgaris*, *A. xanthochlora*, *A. diademata* and *A. rizeensis* are mainly species that have investigated on their antimicrobial and antioxidant activities [10-14]. It has also been reported that *A. mollis* has strong antioxidant, antimicrobial and anti-inflammatory activities [15-17].
To date cytotoxic activity of this plant has not been documented well. Therefore three extracts (50% methanol, water and deodorized water) of *A. mollis* which grown in Rize (Black Sea Region of Turkey) were investigated for its cytotoxic activities on MCF7 cell line. In this study, we have also determined the antiradical and antimicrobial activities of *A. mollis*.

2. Materials and methods
2.1 Plant material and reagents
A. mollis were collected from Rize in Black Sea Region during June and plant specimens were identified and stored at Anadolu University, Herbarium of Faculty of Pharmacy (ESSE 10843). Ultrapure water (18.2 MΩcm) was used throughout and was prepared using a

Millipore Milli-RO 12 plus system (Millipore Corp., MA). All standards and reagents were of the highest purity available and obtained from the Sigma Chemical Co. (St. Louis, MO). All microorganism isolates (except *Klebsiella pneumoniae*) and MCF7 cell line were obtained from the collection of American Type Culture Collection (ATCC).

2.2 Preparation of the extracts

Air-dried *A. mollis* herb material (100.0 g) was sequentially extracted with hexane, ethyl acetate, methanol, and 50% methanol using a Soxhlet apparatus for 8 h for each. Thereafter, the extract was filtered and evaporated to dryness in vacuum at 40 °C. Separately *A. mollis* herb material was extracted with water using reflux for 3 h. The water phase was filtered and freeze-dried. Deodorized water extract of *A. mollis* was prepared using Clevenger apparatus and added and the water phase was filtered and freeze dried. All the extracts were stored at –20 °C. Prior to analysis, an aliquot of each extract was dissolved and filtered through a 0.20 (sterile) and 0.45 µm membrane filter (Whatman, UK) and used for antimicrobial, antioxidant and cytotoxicity assays, resp. Only polar fractions 50% methanol, water and deodorized water extracts were tested. Non polar extracts were not able to use in polar culture medium because of their solubility problems.

2.3 Total phenolic, flavonoid, and flavonols contents

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg of gallic acid/$g_{extract}$ [18]. To ca. 6.0 mL of H_2O, 100.0 µL of sample was transferred in a 10.0 mL volumetric flask, to which 500.0 µL undiluted Folin-Ciocalteu reagent was added subsequently. After 1 min, 1.5 mL 20% (w/v) Na_2CO^3 was added and the volume was made up to 10.0 mL with H_2O. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. The data are presented as the average of triplicate analyses

Total flavonoids were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract}$ [19]. One milliliter of plant extract in methanol (10.0 g/L) was mixed with 1 mL of aluminum trichloride in ethanol (20.0 g/L) and diluted with ethanol to 25.0 mL. The absorption at 415 nm was read after 40 min at 20 °C. Blank samples were prepared from 1 mL plant extract and 1 drop of acetic acid, and diluted to 25.0 mL. The rutin calibration curve was prepared in ethanolic solutions with same procedure. All determinations were carried out in quadruplicate and the mean values were used.

Total flavonols were estimated as rutin equivalents (RE), expressed as $mg_{rutin}/g_{extract.}$ The rutin calibration curve was prepared by mixing 2.0 mL of 0.5–0.015 mg/mL of rutin ethanolic solutions with 2.0 mL (20.0 g/L) of aluminum trichloride and 6.0 mL (50.0 g/L) of sodium acetate. The absorption at 440 nm was read after 2.5 h at 20 °C. The same procedure was carried out with 2.0 mL of plant extract (10.0 g/L) instead of rutin solution. All determinations were carried out in quadruplicate and the mean values were used.

2.4 Antimicrobial assay

Antimicrobial activity testing was performed using standard strains of *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 90028 and isolated strain of *K. pneumoniae* by agar dilution method according to Clinical and Laboratory Standards Institute [20]. The strains were inoculated onto blood agar (Merck, Germany) for bacteria and onto Sabouraud Dextrose Agar (Acumedia, USA) for *C. albicans* from glycerol stocks, incubated at 35 ºC. The microorganism suspensions equivalent to the density of 0.5 McFarland (10^8 CFU/mL for bacteria, 1-5 x 10^6 CFU/mL for yeast) were prepared by comparing density standard (Phoenix Spec Nephelometer, Becton Dickinson, USA) from fresh subcultures in Mueller-Hinton Broth (MHB) (Fluka, BioChemica, Germany). Then bacteria suspensions were diluted to 10^7 CFU/mL using MHB.

Each extract of *A. mollis* was dissolved in distilled water and sterilized by filtration through a membrane filter with 0.2 µm diameter. A stock solution of each extract was diluted in distilled water and 1.0 mL of each dilution was incorporated in 20.0 mL of melted Mueller-Hinton Agar (Merck, Germany). It was obtained the final concentrations of the extracts in the medium ranging from 0.1-10.0 mg/mL. Then, inoculations were performed by means of spots containing 104 CFU/spot from each adjusted microorganism inoculums. The plates were incubated at 35 °C, 18-24 hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentrations of plant extracts inhibiting the visible growth. All determinations were performed twice and two growth controls consisting of medium without extract were included. Mean results were evaluated.

2.4 1,1-Diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging activity

The ability of the extracts to scavenge DPPH• radicals was determined by the method of Gyamfi *et al* [21]. A 50.0 µL aliquot of each extract, in 50.0 mM Tris-HCl buffer (pH 7.4), was mixed with 450 µL of Tris-HCl buffer and 1.0 mL of 0.1 mM DPPH● in MeOH. After 30 min incubation in darkness and at ambient temperature, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using Eq 1. Estimated IC_{50} values are presented as the average of quadruplicate analyses.

2.5 2,2'-Azinobis-3-Ethylbenzothiazoline-6-Sulphonic Acid ($ABTS^{+\bullet}$) Radical Scavenging Activity

This assay assesses the capacity of a compound to scavenge the stable ABTS radical ($ABTS^{\bullet+}$), in comparison to the antioxidant activity of Trolox, a water soluble form of vitamin E that is used as a standard. The blue-green $ABTS^{\bullet+}$ was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate in the dark at room temperature for 12-16 h before use. The concentrated $ABTS^{\bullet+}$ solution was diluted with ethanol to a final absorbance of 0.8-0.7 at 734 nm. A 10.0 µL portion of sample (concentrations of 1.0 and 0.1 mg/mL) was added to 990.0 µL of $ABTS^{\bullet+}$ solution, and the reduction in absorbance was measured 1min after addition of Trolox (final concentration 0.1-2.0 mM) and up to 30 min after addition of the tested compounds [22].

2.6 Cell Line and Cell culture method

The MCF 7 breast cancer cell line was purchased from American Type Culture Collection (ATCC HTB®-22™ Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS), 100 µg/L streptomycin, and 100 IU/mL penicillin at 37 °C in a 5% CO_2 atmosphere.

2.7 The Sulforhodamine B (SRB) Colorimetric Cell Viability Assay on MCF 7 Cell Line

Cells were inoculated into 96-well microtiter plates (1.0×10^4 in 100 µL). After cell inoculation, the plates were incubated for 24 h prior to the addition of extracts. Extracts were

prepared at a 4 mg/mL concentration in cell culture medium containing 1% DMSO. After extract addition at a final concentration of 15.625; 31.25; 62.5; 125; 250; 500; 1000 µg/mL, plates were incubated at standard conditions for 48 h and cells were fixed with 40% (w/v) TCA and incubated for 1 h at 4 °C. The plates were washed five times with water and air-dried. A 0.4% (w/v) SRB in 1% acetic acid was added and the staining was performed for 30 min at room temperature. The SRB solution was removed by washing the plates quickly with water and then with 15% v/v acetic acid. The bound SRB was solubilized by adding 100 µL of 10 mM unbuffered Tris-Base, and the absorbance was measured at 540 nm. The values are represented as the mean values of eight measurements [23].

2.8 Statistical Analysis

Data are presented as mean values ± 95% confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Games–Howell comparison test at a level of $p < 0.05$. The Levene test was used to evaluate the variance homogeneity of the groups.

3. Results & Discussion

The 50% methanol, water and deodorized water extracts of *A. mollis* were prepared and used in activity tests and phenolic composition. The results for the fraction yields, total phenols, total flavonoids and total flavonols are presented in Table 1. According to the data presented in Table 1, the 50% MeOH extract contained the highest amount of total phenols and total flavonols while the water extract contained the lowest amounts. The highest yields were obtained also from the 50% MeOH extracts. The deodorized water extract was found to be contain the highest amount of total flavonoid compounds.

In this study, two different free radicals were used to assess the potential free radical-scavenging activities of the *A. mollis* extract and sub-fractions, namely the $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ synthetic free radicals. The stable nitrogen-centered free radicals, $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ are frequently used for the estimation of free radical-scavenging ability [22, 24]. The $ABTS^{+\bullet}$ free radical is commonly used when issues of solubility or interference arise and the use of $DPPH^{\bullet}$ based assays becomes inappropriate [25]. $DPPH^{\bullet}$ is used to estimate the antiradical activities of the plant extract especially those rich in polar compounds. Polar phenolic compounds can donate an electron to the DPPH radical that can be monitored colorimetrically, *viz*., the purple color of radical changes to yellow. This difference can be quantified spectrophotometrically at 517 nm to calculate the antiradical activity of the samples [26, 27]. This interaction indicates its radical scavenging ability in an iron-free system. In cases where the structure of the electron donor is not known (e.g. as in plant extracts), this method can afford data on the reduction potential of the sample, and hence can be helpful in comparing the reduction potential of unknown materials [26]. Cardinal mode of action of natural antioxidants is their ability to scavenge free radicals before they can initiate free radical chain reactions in cellular membranes or lipid-rich matrices in foodstuffs, cosmetics or pharmaceutical preparations. According to the Figure 1, the IC_{50} values were found 0.264 ± 0.028 mg/mL, 0.146 ± 0.015 and 0.161 ± 0.018 mg/mL respectively for water, deodorized water and 50% MeOH extracts. According to the results of the Levene statistic applied to test variance homogeneity, no assumption was made for the variable ($p < 0.001$). With regard to the results of the Games–Howell test, which was used to compare the groups in multiple comparisons, the activities of BHT and deodorized water extract are statistically the same ($p > 0.05$) while their activities are found statistically significant than the activities of water and 50% MeOH extracts ($p < 0.05$).

As can be seen in Figure 2, all extracts and positive control manage to inhibit the $ABTS^{\bullet+}$ radical. The TEAC values of water extract (0.90 ± 0.01 and 1.55 ± 0.02 mM/L/Trolox) were found higher than the deodorized water and 50% MeOH extracts ($p < 0.05$). According to the results of the Levene statistic, hypothesis for the variable was not provided ($p < 0.001$). Activities of BHT and water extract are statistically the same ($p > 0.05$). According to the results of the Games–Howell test, there was no statistically significant difference ($p > 0.05$) between the activities of 50% MeOH and water extracts in both concentrations. Both water and deodorized water extracts of *A. mollis*, showed the most reductive activity and were also found to be good radical scavengers.

The extracts of *A. mollis* were tested against clinically important pathogens including *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *C. albicans.* The results of the antimicrobial activity of all the extracts against tested microorganisms are presented in Table 2. With the exception of *C. albicans*, the extracts demonstrated moderate activity against the Gram-positive and Gram-negative bacteria tested in this study and the MIC ranged from 0.5 to 7.5 mg/mL. All the extracts showed better activity on *S. aureus* which is known pathogen commonly associated with skin and mucosa infections and syndromes associated with toxins and invasive diseases (MIC = 0.5 mg/mL). *C. albicans* strains were resistant to all extracts. Comparison of the MIC values of extracts shows that all extracts have similar activities against each bacteria. *A. mollis* contains hydrolysable tannins such as gallotannins and ellagitannins as effective compounds against to bacteria. According to the literature, gallotannins inhibited the *S. aureus* but not the intestinal Gram negative bacteria such as *E. coli* [28-30]. *A. vulgaris* and some other species shown different degrees of antibacterial activity against the *S. aureus* and not inhibited the *E. coli*. In our study, *S. aureus* was also inhibited by the polar extracts of *A. mollis* contained polyphenolics such as tannins.

The results obtained by SRB cell viability method are given in Figure 3. The multiple concentrations of extracts were used and effective doses were calculated from dose-response curve. Results clearly demonstrated that the water extract of *A. mollis* has the highest cytotoxic effect among the other extracts. Water extract was significantly cytotoxic between the 62.5-1000 µg/mL concentrations ($p < 0.05$). The IC_{50} value of the water, deodorized water and methanol extracts are 59.34 ± 3.41 µg/mL, 87.37 ± 25.15 µg/mL, 68.18 ± 6.12 µg/mL, respectively. Total phenolic content of the extracts to be compatible with cytotoxic IC_{50} values suggest that phenolic content is effective on the cytotoxic activity. The antioxidant mechanisms of phenolics include scavenging the free radicals to terminate the radical chain reaction, absorbance of oxygen radicals (ROS), chelating transition metals, interfering with the enzymes ROS producing and stimulating the anti-oxidative enzyme activities, thus they decreased the incidence of cancer. In addition, they have been identified as anti-proliferative agents due to their ability to cell cycle arrest, induce apoptosis, destruction mitotic spindle formation and inhibit angiogenesis [31]. This is the first study to reveal the cytotoxic effects of *A. mollis* on MCF7 cell line.

3.1 Tables and Figures

Table 1: The amounts of yield, total phenolics, total flavonoids, and total flavonols of *Alchemilla mollis*

Samples	Yield [%]	Total Flavonols [$mg_{RE}/g_{extract}$][a]	Total Flavonoids [mg_{RE}/g_{etract}]	Total Phenols [mg_{GAE}/g_{etract}][b]
Water extract (WE)	6.44	9.8 ± 0.3[c]	47.4 ± 0.3	281.8 ± 4.8
Deodorized water extract (DWE)	8.34	15.8 ± 0.6	50.0 ± 1.1	285.7 ± 8.1
Methanol (50%) extract (ME)	1.62	27.4 ± 1.0	46.4 ± 5.9	332.7 ± 4.8

[a][$mg_{RE}/g_{extract}$], milligram rutin equivalent /gram extract; [b][$mg_{GAE}/g_{extract}$], milligram gallic acid equivalent /gram extract; [c]mean ± SD

Table 2: *In vitro* antimicrobial activities of extracts of *Alchemilla mollis*.

Microorganisms	Minimum inhibitory concentration (MIC, mg mL^{-1})		
	ME[a]	WE	DWE
Staphylococcus aureus	0.5	0.5	0.5
Escherichia coli	-	5.0	5.0
Pseudomonas aeruginosa	2.0	2.0	2.0
Enterococcus faecalis	5.0	7.5	7.5
Klebsiella pneumoniae	5.0	7.5	7.5
Candida albicans	-	-	-

[a]ME, 50% methanol extract; WE, water extract; DWE, deodorized water extract

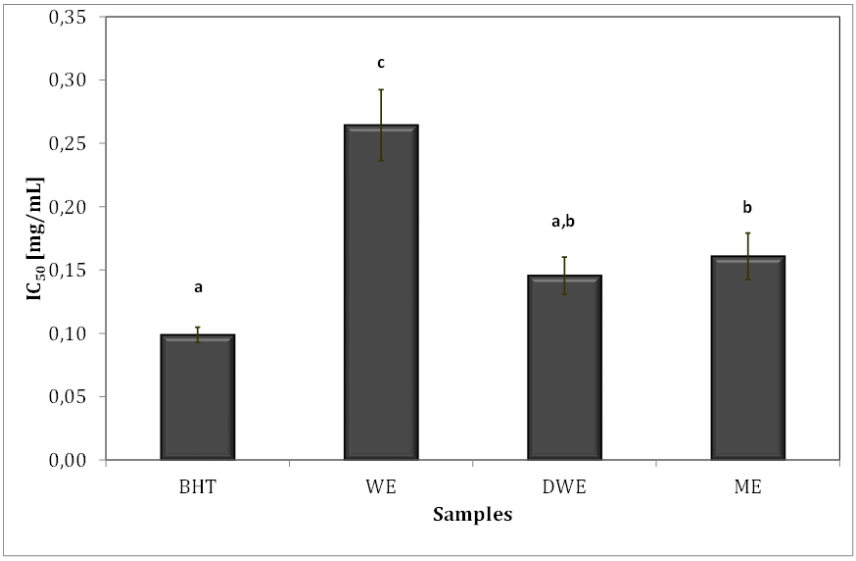

Fig 1: DPPH radical scavenging activity of *Alchemilla mollis* extracts (BHT, butylated hydroxytoluene; ME, 50% methanol extract; WE, water extract; DWE, deodorized water extract). Values = mean ± SD, statistical analyses by Games–Howell comparison test, bars with the same lower case letter (a – c) are not significantly ($p > 0.05$) different, n = 3.

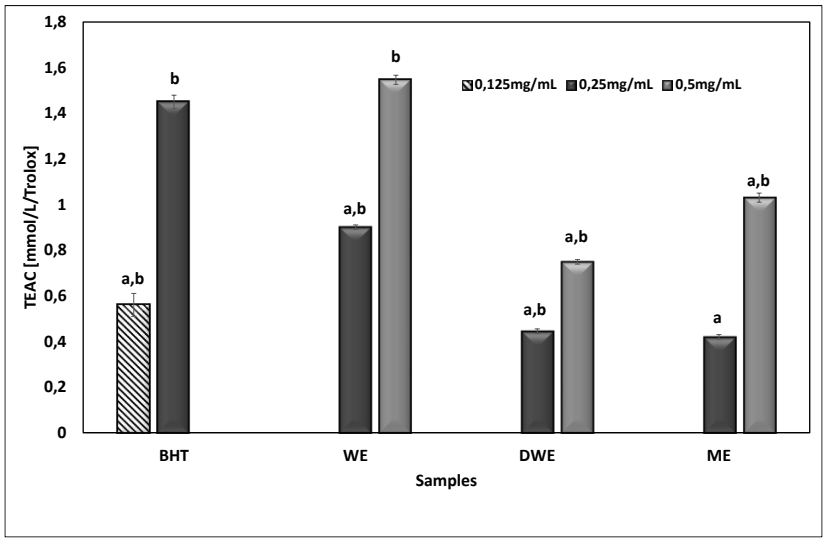

Fig 2: $ABTS^{+}$ radical scavenging activity of *Alchemilla mollis* extracts in two concentrations (BHT, butylated hydroxytoluene; ME, 50% methanol extract; WE, water extract; DWE, deodorized water extract). Values = mean ± SD, statistical analyses by Games–Howell comparison test, bars with the same lower case letter (a – b) are not significantly ($p > 0.05$) different, n = 3.

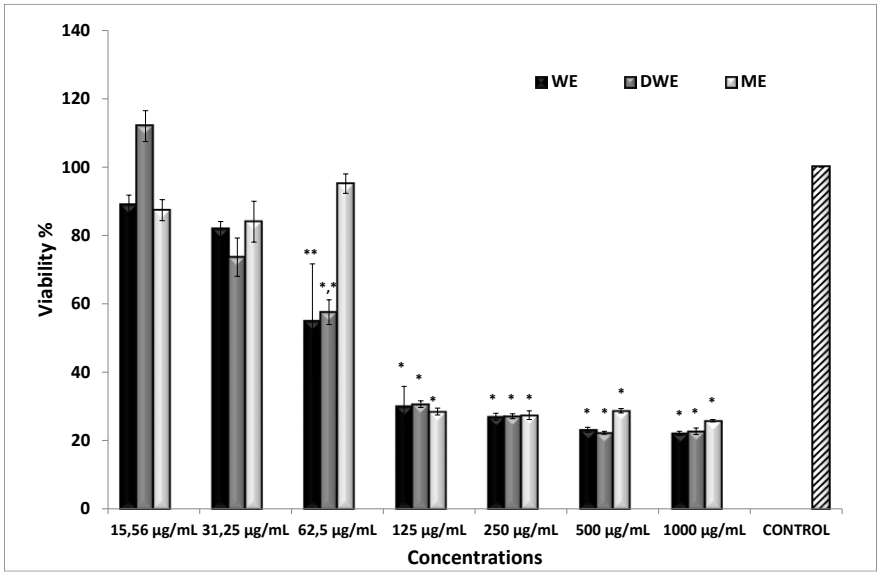

Fig 3: Cytotoxic effect of *Alchemilla mollis* extracts on MCF 7 cell line. (ME, 50% methanol extract; WE, water extract; DWE, deodorized water extract) Values = mean ± SD, statistical analyses by Games–Howell comparison test, levels of significance are indicated as *p<0.001,**p<0.05

3.2 Equations

$$\text{Percentage Inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] x100$$

Equation 1.

4. Conclusions

In conclusion, all extracts of *Alchemilla* assessed in this study show antiradical, antimicrobial and cytotoxic activities. All the extracts contained varying amounts of phenolic compounds, as estimated by the Folin-Ciocalteau reagent method. *Alchemilla* species have gallotannins and ellagitannins, also flavonoids, according to the literature. These polyphenolics were responsible for antiradical, antimicrobial and cytotoxic activities. Plant derived hydrolysable tannins have potential as new and safe therapeutic regimens against *S. aureus* infections and the activity of the extracts on *S. aureus* confirm the traditional use of the plant in skin infections. The cytotoxic activity on MCF7 cell line of the *A. mollis* was first studied by us and the results are promising. Further studies are needed to confirm *in vivo* anti-tumorgenicity and subsequent chemical characterization of the active molecule(s).

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