

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Original Article

Extracts of *Agrimonia eupatoria* L. as sources of biologically active compounds and evaluation of their antioxidant, antimicrobial, and antibiofilm activities



Mirjana Ž. Muruzović*, Katarina G. Mladenović, Olgica D. Stefanović,
Sava M. Vasić, Ljiljana R. Čomić

Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića, Kragujevac, Serbia

ARTICLE INFO

Article history:

Received 12 October 2015

Received in revised form

2 February 2016

Accepted 26 February 2016

Available online 25 April 2016

Keywords:

Agrimonia eupatoria
antimicrobial activity
antioxidants
biofilm
flavonoids
phenolics
tannins

ABSTRACT

In this study, we determined the concentration of total phenols, flavonoids, tannins, and proanthocyanidins in the water, diethyl ether, acetone, and ethanol extracts of *Agrimonia eupatoria* L. We also investigated the antioxidant activity of these extracts using two methods [2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power] and their *in vitro* antimicrobial (antibacterial and antifungal) activity on some selected species of bacteria and fungi. In addition, the effects of the acetone and water extracts on the inhibition of biofilm formation of *Proteus mirabilis* and *Pseudomonas aeruginosa* were investigated using the crystal violet method. The concentration of total phenols was measured according to the Folin–Ciocalteu method and the values obtained ranged from 19.61 mgGA/g to 220.31 mgGA/g. The concentration of flavonoids was examined by the aluminum chloride method and the values obtained ranged from 20.58 mgRU/g to 97.06 mgRU/g. The total tannins concentration was measured by the polyvinylpyrrolidone method and the values obtained ranged from 3.06 mgGA/g to 207.27 mgGA/g. The concentration of proanthocyanidins was determined by the butanol–HCl method and the values obtained ranged from 4.15 CChE/g to 103.72 CChE/g. Among the various extracts studied, the acetone extract exhibited good antioxidant activity (97.13%, as determined by the DPPH method). The acetone extract was active in the absorbance value range from 2.2665 to 0.2495 (as determined by the reducing power method). The strongest antimicrobial activity was detected on G⁺ bacteria, especially on probiotic species, and the acetone extract demonstrated the highest activity. Biofilm inhibitory concentration required to reduce biofilm coverage by 50% values for acetone extract was 4315 µg/mL for *P. mirabilis* and 4469.5 µg/mL for *P. aeruginosa*. The results provide a basis for further research of this plant species.

Copyright © 2016, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author. Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia.

E-mail address: mirkagrujovic@gmail.com (M.Ž. Muruzović).

<http://dx.doi.org/10.1016/j.jfda.2016.02.007>

1021-9498/Copyright © 2016, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Agrimonia eupatoria L. (common name: agrimony) belongs to the family Rosaceae (Tribe: Sanguisorbeae). The species is widespread throughout Europe, Asia, Africa, and North America. The common habitats of this species are along the slopes, roadsides, and rocky areas. They are also found in dry grasslands and arid forests. Agrimony is a perennial herbaceous plant characterized by upright, hairy stem with a few branches. The leaves are leathery, plumose, and the lower ones frequently form a rosette. The flowers are arranged in thick, spiky bunches. The fruit grows downward [1].

The plant is well-known for its use as a raw material for the extraction of medicinal ingredients or production of drugs in the pharmaceutical industry. A tea made from agrimony helps to cure digestive tract diseases. The plant is also an important ingredient of various herbal mixtures that are used as dietary supplements for treating increased bile production, stone in the bile duct, and pain in the gallbladder and liver; additionally, it has a positive effect on the alleviation of urinary tract disorders [2]. According to previous studies, *A. eupatoria* is very rich in secondary metabolites, including tannins, flavonoids, phenolic acids, and triterpenoids [3–7]. The plant is recognized for its traditional use and has been considerably studied. The plant possesses anti-inflammatory, neuroprotective, antidiabetic, antiobesity, hepatoprotective, and anticancer properties [8–13]. A high correlation between polyphenolic contents and antioxidant activity in the water–alcoholic extracts of *A. eupatoria* was detected [14]. The antioxidant activity of agrimony water extracts has been demonstrated both *in vivo* and *in vitro* by measuring the changes in the activities of hepatic catalase and superoxide dismutase in mice [15]. A recent study on the tea prepared from *A. eupatoria*, which was given to healthy volunteers, confirmed the antioxidant properties of the plant [16]. The antiviral properties of the water extract prepared from ground parts at four different temperatures (37°C, 45°C, 55°C, and 60°C) were tested on hepatitis B virus and it was concluded that the extract prepared at 60°C had the strongest antiviral effect [17]. In another clinical study, herbal preparations containing agrimony were successfully used for treating 35 patients suffering from gastro-duodenitis. Moreover, there was no documented side effects or signs of toxicity [18]. *A. eupatoria* is a well-known medicinal plant, which is traditionally used in folk medicine to treat various inflammatory diseases. Although it is known that the plant synthesizes secondary metabolites that exhibit antimicrobial activity, only limited studies were carried out in this regard. Besides, some studies have clearly demonstrated the antioxidant activity of water and ethanol extracts of this plant; however, the activity of acetone and diethyl ether extracts of this plant has not yet been studied. Moreover, the antibiofilm activity of these extracts is yet to be elucidated.

In this study, we aimed to determine the concentration of total phenols, flavonoids, tannins, and proanthocyanidins in the water, diethyl ether, acetone, and ethanol extracts of *A. eupatoria*. We also investigated the antioxidant activity of these extracts using two methods [2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power] and their *in vitro* antimicrobial (antibacterial and antifungal) activity on some selected species

of bacteria and fungi. In addition, the effects of the acetone and water extracts on the inhibition of biofilm formation of *Proteus mirabilis* and *Pseudomonas aeruginosa* were investigated.

2. Methods

2.1. Plant material

The aerial parts of *A. eupatoria* in the flowering stage were collected from Mount Bukulja (Serbia) during the summer of 2012. Identification and classification of the plant material were performed at the Faculty of Science, University of Kragujevac (Kragujevac, Serbia). The voucher samples were deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant materials were air dried in darkness at ambient temperature.

2.2. Preparation of plant extracts

The dried, ground plant material was extracted by maceration with ethanol, diethyl ether, water, and acetone. In brief, 30 g of the plant material was soaked in 150 mL of the solvent. The plant material was macerated three times at room temperature using a fresh solvent every 24 hours. After every 24 hours, the samples were filtered through a filter paper (Whatman No. 1) and the filtrates were collected and evaporated to dryness using a rotary evaporator (IKA, Germany) at 40°C. The obtained amounts of crude extracts of *A. eupatoria* were 5.42 g of water extract, 0.55 g of diethyl ether extract, 0.76 g of acetone extract, and 2.4 g of ethanol extract.

2.3. Phytochemical analysis of plant extracts

2.3.1. Determination of total phenol content

The total phenolic content of the extracts was quantified according to the Folin–Ciocalteu method as described by Wootton-Beard et al [19]. Gallic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the total phenolic content was expressed as milligram of gallic acid equivalents/gram of extract (mg GAE/g of extract).

2.3.2. Determination of total flavonoid content

The total flavonoid content of the extracts was determined using the aluminum chloride method as described by Quettier-Deleu et al [20]. Rutin (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the concentrations of flavonoids were expressed as milligram of rutin equivalents/gram of extract (mg of RUE/g of extract).

2.3.3. Determination of total extractable tannin content

The total extractable tannin content was estimated indirectly by spectrophotometric measurement of the absorbance of the solution obtained after the precipitation of the tannins with polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO, USA) as described by Makkar et al [21]. The total extractable tannin content was expressed as milligram of gallic acid equivalents/gram of extract (mg GAE/g of extract).

2.3.4. Determination of proanthocyanidin content

The proanthocyanidin content was measured by the butanol–HCl method with ferric ammonium sulfate as a catalyst as described by Porter et al [22]. Cyanidin chloride (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the proanthocyanidin content was expressed as milligrams of cyanidin chloride equivalents/gram of extract (mg CChE/g of extract).

2.4. Determination of antioxidant activity

2.4.1. DPPH radicals scavenging capacity assay

The ability of *A. eupatoria* extracts to scavenge DPPH free radicals was assessed using the method described by Takao et al [23]. The tested concentrations of plant extracts were from 7.8 µg/mL to 250 µg/mL. Diluted solutions of extract (2 mL each) were mixed with 2 mL of DPPH methanolic solution (40 µg/mL). Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as a reference compound. The experiment was performed in triplicate, and the absorbance of reaction mixture was read in a spectrophotometer at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following formula:

$$\text{Scavenging activity (\%)} = 100 \times [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}](1)$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the extract.

2.4.2. Reducing power

The reducing power of the plant extracts was determined according to the method of Oyaizu [24]. The tested concentrations of plant extracts were from 62.5 µg/mL to 1000 µg/mL. The absorbance of the reaction mixture was measured at 700 nm, with each experiment performed in triplicate. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference compound.

2.5. Determination of antimicrobial activity

2.5.1. Test microorganisms

The antimicrobial activity of *A. eupatoria* extracts was tested against 24 microorganisms including 18 strains of bacteria (probiotics strains: *Lactobacillus rhamnosus*, *Bifidobacterium animalis* subsp. *lactis*, and *Bacillus subtilis* IP 5832; standard strains: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *B. subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, and *P. aeruginosa* ATCC 27853; and clinical isolates *S. aureus*, *E. faecalis*, *Bacillus cereus*, *B. subtilis*, *E. coli*, *Salmonella enterica*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *P. mirabilis*, and *P. aeruginosa*) and six strains of fungi (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium italicum*, *Penicillium chrysogenum*, *Candida albicans*, and *C. albicans* ATCC 10231). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The other microorganisms (fungi and ATCC strains) were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at -80°C and the fungal strains in paraffin oil stock at 4°C .

2.5.2. Suspension preparation

Bacterial and yeast suspensions were prepared by the direct colony method [25]. The turbidity of initial suspension was adjusted using 0.5 McFarland densitometer (Biosan, Latvia). Initial bacterial suspensions contain about 10^8 colony-forming units (CFU)/mL and yeast suspensions contain 10^6 CFU/mL; 1:100 dilutions of initial suspension were additionally prepared in sterile 0.85% saline. The suspensions of fungal spores were prepared by gentle stripping of spores from slopes with growing mycelia. The resulting suspensions were 1:1000 diluted in sterile 0.85% saline.

2.5.3. Microdilution method

The antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) using the microdilution method with resazurin [26]. Twofold serial dilutions of the plant extracts were made in sterile 96-well microtiter plates containing 0.1 mL of Mueller–Hinton broth (Torlak, Belgrade, Serbia) per well for bacteria and 0.1 mL of Sabouraud dextrose broth (Torlak, Belgrade, Serbia) per well for fungi. The tested concentration range was from 0.156 mg/mL to 20 mg/mL. The microtiter plates were inoculated with the suspensions to obtain a final concentration of 5×10^5 CFU/mL for bacteria and 5×10^3 CFU/mL for fungi. The growth of bacteria and yeasts was monitored by adding resazurin (Alfa Aesar GmbH & Co., Karlsruhe, Germany), an indicator of microbial growth. Resazurin is a blue nonfluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated microtiter plates were incubated at 37°C for 24 hours for bacteria, at 28°C for 48 hours for yeasts, and at 28°C for 72 hours for molds. MIC was defined as the lowest concentration of tested plant extracts that prevented resazurin color change from blue to pink. For molds, MIC values of the tested plant extracts were determined as the lowest concentration that inhibited visible mycelia growth. Minimum microbicidal concentration (MMC) was determined by inoculation of the nutrient agar medium by plating 10 µL of samples from wells, where no indicator color change was recorded. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as MMC.

Tetracycline, ampicillin, amphotericin B (Sigma Chemicals Co., USA), and itraconazole (Pfizer Inc., USA), dissolved in the nutrient liquid medium, were used as reference compounds. Stock solutions of crude extracts were obtained by dissolving them in 10% dimethyl sulfoxide (DMSO; Acros Organics, USA), which was used as a control. Each test included growth control and sterility control. All tests were performed in duplicate and mean values were presented.

2.6. Determination of antibiofilm activity

2.6.1. Tissue culture plate method

The ability of *P. mirabilis* and *P. aeruginosa* to form biofilms was assayed as described by O'Toole and Kolter [27] with some modifications.

The tissue culture 96-well microtiter plates (Sarstedt, Germany) were prepared by dispensing 50 µL of nutrient broth (i.e., Mueller–Hinton broth) into each well. From the stock

solution of tested extracts (concentration, 20 mg/mL), 50 μ L was added into the first row of the microtiter plate. Twofold serial dilutions were then made using a multichannel pipette, following which 50 μ L of fresh bacterial suspension was added into each well. The inoculated microtiter plates were incubated at 37°C for 24 hours. After incubation, the content of each well was gently removed by tapping the microtiter plates. The wells were washed with 200 μ L of sterile 0.85% saline to remove free-floating bacteria. Biofilms formed by adherent cells in 96-well microtiter plates were stained with crystal violet (0.1% w/v; Fluka AG, Switzerland) and incubated at the room temperature for 20 minutes. Excess stain was rinsed off by thorough washing with deionized water and then with 200 μ L of 96% ethanol. Optical densities (ODs) of stained adherent bacteria were determined with an enzyme-linked immunosorbent assay (ELISA) plate reader (RT-2100C, Rayto, Shenzhen, China) at 630 nm wavelength. Biofilm inhibitory concentration required to reduce biofilm coverage by 50% (BIC₅₀) was defined as the lowest concentration of extract that showed 50% inhibition on the biofilm formation [28].

Only broth or broth with extracts served as control to check sterility and nonspecific binding of media. To compensate for background absorbance, OD readings from sterile medium, extracts, fixative, and dye were averaged and subtracted from all test values. All tests were performed in duplicate. Tetracycline dissolved in nutrient liquid medium was used as the reference compound.

2.6.2. Data analysis

All data were presented as means \pm standard deviations where appropriate. Pearson correlation coefficients were determined using Microsoft Excel (Redmond, Washington, DC, USA). For comparison of antibacterial activity between groups of bacteria (G⁻, G⁺, and probiotics), data were analyzed by one-way analysis of variance using SPSS version 20 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. The contents of total phenols, flavonoids, total extractable tannins, and proanthocyanidins

Secondary metabolites such as flavonoids, phenols, tannins, and proanthocyanidins possess a high solubility in water, acetone, ethanol, and diethyl ether and this is the criterion for selecting solvent for plant material extraction. The results are shown in Table 1. The acetone extract showed the maximum measured concentration of total flavonoids (97.06 mgRU/g), total phenols (220.31 mgGA/g), total extractable tannins (207.27 mgGA/g), and total proanthocyanidins (103.72 CChE/g). The concentrations in ethanol and water extracts were half as much. The diethyl ether extract had the lowest concentration of total phenolic content, total tannins, and proanthocyanidins.

Based on these results, it was concluded that the highest concentration of secondary metabolites was in the acetone extract, whereas the lowest was in the diethyl ether extract. The reason for the highest concentration in the acetone

extract might be because acetone is an effective extractant with low toxicity and high extraction capacity [29].

3.2. Antioxidant activity

3.2.1. DPPH radical scavenging activity

The extracts and vitamin C used as a positive control were tested in the concentration range from 7.8 μ g/mL to 250 μ g/mL (Table 2). The antioxidant activity was highest for the acetone extract and ranged from 97.13% to 27.73%. The ethanol and water extracts also demonstrated a significant antioxidant activity. Diethyl ether extract had the lowest antioxidant activity (53.1–14.3%). Vitamin C was active in the range from 97.18% to 55.01%.

In this study, correlation between phenolic compounds contents and DPPH radical scavenging activity was observed. Positive linear correlation was shown for the total phenolic, tannin, and proanthocyanidin contents ($r = 0.85, 0.86,$ and $0.91,$ respectively), whereas there was a negative linear correlation for flavonoids ($r = -0.09$).

Based on these results, it was concluded that higher concentrations of acetone extract and vitamin C operated in a similar way, confirming the high antioxidant activity of agrimony. All tested extracts showed a concentration-dependent antiradical activity.

3.2.2. Reducing power

The reducing power of the extracts is related to their electron-donating ability and may serve as a significant indicator of potential antioxidant activity. The extract concentrations from 62.5 μ g/mL to 1000 μ g/mL were tested and compared with the results gained for the equal concentrations of vitamin C, which was used as a positive control (Table 3). The extent of reducing the power of examined extracts was found to vary. Among the various extracts studied, the acetone extract was found to be the most active, followed by ethanol, water, and diethyl ether extracts (acetone > ethanol > water > diethyl ether). The acetone extract was active in the absorbance range from 2.2665 to 0.2495, ethanol extract from 1.6172 to 0.0868, water extract from 0.9475 to 0.1162, and diethyl ether extract from 0.5059 to 0.0537. Ascorbic acid (vitamin C) was active in the absorbance range from 2.943 to 1.19.

The correlation between phenolic compounds contents and reducing power showed a linear correlation in relation to the total phenolic, flavonoid, tannin, and proanthocyanidin contents ($r = 0.94, 0.56, 0.93,$ and $0.96,$ respectively).

On the basis of the obtained results, it was concluded that the examined extracts of *A. eupatoria* showed moderate reducing power compared with the positive control, and they showed activity in all examined concentrations. It was also concluded that the reducing power depends on concentration. To the best of the authors' knowledge, the reducing power of *A. eupatoria* extracts has not been investigated before.

3.3. Antimicrobial activity

The results of *in vitro* antibacterial and antifungal activities of water, acetone, diethyl ether, and ethanol extracts of *A. eupatoria*, determined by MICs and MMCs, are shown in Tables 4 and 5. A total of 24 species of microorganisms were tested

Table 1 – Concentrations of flavonoids, total phenolic content, total extractable tannins, and proanthocyanidins in the extracts of *Agrimonia eupatoria*.

Type of extract	Flavonoid concentration (mgRU/g extract)	Total phenolic content (mgGA/g extract)	Total extractable tannins concentration (mgGA/g extract)	Concentration of proanthocyanidins (CChE/g extract)
Water	20.58 ± 0.92	118.47 ± 0.72	107.52 ± 0.16	55.85 ± 0.75
Acetone	97.06 ± 2.56	220.31 ± 0.00	207.27 ± 0.21	103.72 ± 0.53
Diethyl ether	64.9 ± 0.79	19.61 ± 0.10	3.06 ± 5.98	4.15 ± 0.41
Ethanol	46.5 ± 0.08	123.9 ± 0.47	109.33 ± 0.09	74.42 ± 0.73

Data are presented as mean ± standard deviation.

Table 2 – Comparison of antioxidant activity of different *Agrimonia eupatoria* extracts obtained by the 2,2-diphenyl-1-picrylhydrazyl method.

Concentration (µg/mL)	Type of extract				
	Water extract	Acetone extract	Diethyl ether extract	Ethanol extract	Ascorbic acid
250	93.95 ± 0.27	97.13 ± 0.26	53.1 ± 0.40	94.88 ± 0.14	97.18
125	92.87 ± 0.46	94.83 ± 0.22	30.46 ± 0.18	94.79 ± 0.13	97.18
62.5	90.25 ± 1.27	91.98 ± 0.24	19.6 ± 0.31	94.08 ± 0.16	97.15
31.25	71.86 ± 0.25	87.57 ± 0.04	9.7 ± 0.40	59.59 ± 0.07	97.06
15.6	40.91 ± 0.29	50.77 ± 0.53	6.88 ± 0.44	33.88 ± 0.10	92.01
7.8	21.9 ± 0.22	27.73 ± 0.36	3.14 ± 0.50	19.4 ± 0.43	55.01

Data are presented as mean ± standard deviation. Values represent % of the activity.

Table 3 – Comparison of reducing power of various *Agrimonia eupatoria* extracts and ascorbic acid.

Concentration (µg/mL)	Type of extract				
	Water extract	Acetone extract	Diethyl ether extract	Ethanol extract	Ascorbic acid
1000	0.9475 ± 0.00	2.2665 ± 0.00	0.5059 ± 0.00	1.6172 ± 0.09	2.943
500	0.6899 ± 0.00	1.2968 ± 0.02	0.2789 ± 0.00	0.8843 ± 0.01	2.943
250	0.3759 ± 0.00	0.7859 ± 0.05	0.1703 ± 0.00	0.3603 ± 0.13	2.843
125	0.2682 ± 0.00	0.4614 ± 0.00	0.0941 ± 0.00	0.1169 ± 0.00	2.667
62.5	0.1162 ± 0.00	0.2495 ± 0.00	0.0537 ± 0.00	0.0868 ± 0.00	1.190

Data are presented as absorbance mean value ± standard deviation measured at 700 nm.

and the results were compared with the influence of ampicillin and tetracycline for bacteria and with itraconazole and amphotericin B for fungi. Tetracycline is a broad-spectrum antibiotic effective against aerobic and anaerobic G⁺ and G⁻ bacteria. Ampicillin is a beta-lactam antibiotic that attacks G⁺ and some G⁻ bacteria. Amphotericin B is an antifungal drug often used for serious systemic fungal infections and is one of the effective treatments for fungal infections caused by *Aspergillus* sp. Itraconazole prevents the growth of *Candida* sp. In our experiments, it was observed that 10% DMSO did not inhibit the growth of microorganisms.

In this experiment, the values of the MIC and MMC were in the range from < 0.156 mg/mL to > 20 mg/mL. The intensity of antibacterial activity depended on the type of extract and the species of bacteria. Extracts were active in the following ascending order: diethyl ether < ethanol < water < acetone.

It was noted that the extracts generally had a weak effect on G⁻ bacteria than on G⁺ bacteria and probiotics ($p < 0.05$; Table 4). *E. coli*, *S. enterica*, and *S. typhimurium* showed resistance to the tested extracts (> 20 mg/mL), whereas *E. faecalis* showed resistance to all the tested extracts except for the acetone extract, which was active in the concentration of

10 mg/mL for MIC and MMC. The acetone extract also showed the highest inhibitory effect on G⁺ and G⁻ bacteria. It was most efficient on G⁺ strains of *L. rhamnosus* and *B. animalis* subsp. *lactis* (MIC and MMC < 0.156 mg/mL), and also on *B. cereus*, *B. subtilis*, and *B. subtilis* ATCC 6633 (MIC and MMC at 0.3125 mg/mL). With regard to G⁻ strains, bacteria most sensitive to the acetone extract were *P. aeruginosa* (MIC at 0.625 mg/mL and MMC at 1.25 mg/mL) and *E. coli* ATCC 25922 (MIC at 1.25 mg/mL and the MMC at 2.5 mg/mL). It has been reported that G⁺ bacteria are usually more sensitive to the plant-origin antimicrobials, compared with G⁻ bacteria, which are usually more resistant. The resistance of the G⁻ bacteria could be attributed to their cell wall structure. G⁻ bacteria have an effective permeability barrier composed of a thin lipopolysaccharide exterior membrane, which could restrict the penetration of the active compounds from plant extracts [30].

The examined fungi showed low sensitivity to the tested extracts (Table 5). The acetone extract was active in the 2.5–20 mg/mL range for MIC and MMC, whereas the diethyl ether, water, and ethanol extracts were active in the 5–20 mg/mL range for MIC and MMC. The effect was more noticeable on

Table 4 – Antibacterial activities of water, acetone, diethyl ether, and ethanol extracts of *Agrimonia eupatoria*.

Species	Water extract		Diethyl ether extract		Acetone extract		Ethanol extract		Tetracycline		Ampicillin	
	MIC	MMC ^a	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
<i>Escherichia coli</i> ATCC 25922	2.5	3.75	2.5	10	1.25	2.5	1.25	2.5	4	6	0.37	0.5
<i>E. coli</i>	>20	>20	>20	>20	10	10	20	>20	2	6	2.1	1.2
<i>Salmonella enterica</i>	>20	>20	>20	>20	10	10	20	20	2	2	1	1
<i>Salmonella typhimurium</i>	>20	>20	>20	>20	7.5	10	10	20	2	2	2	2
<i>Pseudomonas aeruginosa</i> ATCC 27853	10	20	>20	5	2.5	2.5	5	>20	4	32	>128	>128
<i>P. aeruginosa</i>	10	20	20	3.75	0.62	1.25	1.25	>20	>128	>128	>128	>128
<i>Proteus mirabilis</i>	5	20	>20	5	2.5	2.5	2.5	20	>128	>128	>128	>128
<i>Klebsiella pneumoniae</i>	>20	>20	>20	10	10	10	20	>20	4	32	>128	>128
<i>Staphylococcus aureus</i> ATCC 25923	2.5	5	5	5	1.25	2.5	1.25	2.5	1.5	3	0.25	0.75
<i>S. aureus</i>	0.62	2.5	0.31	1.25	0.12	0.62	0.62	1.25	<0.06	<0.06	<0.06	<0.06
<i>Bacillus subtilis</i> ATCC 6633	1.25	5	0.62	1.87	0.31	0.31	0.62	1.25	0.25	0.37	3	4
<i>B. subtilis</i>	1.25	5	0.62	1.25	0.31	0.62	0.62	1.25	<0.06	0.25	16	128
<i>Bacillus cereus</i>	1.25	1.25	0.62	0.62	0.31	0.31	0.62	0.62	0.25	0.5	4	6
<i>Enterococcus faecalis</i> ATCC 29212	5	>20	5	20	2.5	5	1.87	5	8	12	1	2
<i>E. faecalis</i>	>20	>0	>20	>20	10	10	20	20	1	6	4	6
<i>Lactobacillus rhamnosus</i>	1.25	2.5	0.31	0.31	<0.16	<0.16	<0.16	<0.16	0.16	1	64	13
<i>Bifidobacterium animalis</i> subsp. <i>lactis</i>	0.16	1.87	<0.16	<0.16	<0.16	<0.16	<0.16	<0.16	4	8	<0.06	0.12
<i>B. subtilis</i> IP 5832	2.5	2.5	0.62	0.62	0.31	0.31	0.31	0.62	<0.06	<0.06	8	16

MIC = minimum inhibitory concentration; MMC = minimum microbicidal concentration.

^a Data are given as mg/mL for plant extract and µg/mL for antibiotics.

Table 5 – Antifungal activities of water, acetone, diethyl ether, and ethanol extracts of *Agrimonia eupatoria*.

Species	Water extract		Diethyl ether extract		Acetone extract		Ethanol extract		Amphotericin B		Itraconazole	
	MIC	MMC ^a	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
<i>Candida albicans</i> ATCC 10231	20	20	5	5	2.5	5	5	5	0.49	1.95	1.95	1.95
<i>C. albicans</i>	5	5	5	5	5	5	5	5	0.98	1.95	1.95	1.95
<i>Penicillium italicum</i>	20	20	7.5	7.5	2.5	2.5	10	10	7.81	7.81	0.98	0.98
<i>Penicillium chrysogenum</i>	20	20	5	5	2.5	2.5	10	10	7.81	7.81	0.98	0.98
<i>Aspergillus flavus</i>	20	20	20	20	10	10	20	20	0.98	15.62	0.98	0.98
<i>Aspergillus niger</i>	20	20	20	20	20	20	20	20	0.98	0.98	15.62	15.62

MIC = minimum inhibitory concentration; MMC = minimum microbicidal concentration.

^a Data are given as mg/mL for plant extract and µg/mL for antimycotics.

yeasts, than on filamentous fungi. *C. albicans* was partially sensitive to the activity of all the extracts, in the concentration of 5 mg/mL for MIC and MMC, and *C. albicans* ATCC 10231 was the most sensitive to the acetone extract in the concentration of 2.5 mg/mL for MIC and 5 mg/mL for MMC. The acetone extract was the most effective against *P. italicum* and *P. chrysogenum* in the concentration of 2.5 mg/mL for MIC and MMC.

3.4. Antibiofilm activity

Biofilms were quantified by measuring the absorbance of stained biofilms at 630 nm with an ELISA plate reader. The results indicated that *P. aeruginosa* and *P. mirabilis* have the ability to form biofilms better than other bacteria studied. The absorbance values were 1.047 for *P. aeruginosa* and 0.285 for *P. mirabilis* (Table 6).

In vitro activity of water and acetone *A. eupatoria* extracts on the inhibition of biofilm formation was examined. Absorbance values of controls were subtracted from the absorbance values of the tested samples (fixed and dyed) in order to recompense the background absorbance. The absorbances are shown in Table 6. The absorbance values for acetone extract ranged between 1.123 and 0.411 for *P. aeruginosa* and between 0.258 and 0.094 for *P. mirabilis*. The absorbance values for water extract ranged between 1.163 and 0.714 for *P. aeruginosa* and between 0.493 and 0.275 for *P. mirabilis*. Decrease of the absorbance values of tested samples in relation to the absorbance of growth control demonstrated the ability of tested extracts to prevent the biofilm formation.

BIC₅₀ was defined as the lowest concentration of extract that showed 50% inhibition on the biofilm formation. The results are shown in Table 7. The acetone extract showed a

Table 6 – Antibiofilm activity of water and acetone extracts of *Agrimonia eupatoria*.^a

Concentration ($\mu\text{g/mL}$)	Species					
	<i>Pseudomonas aeruginosa</i>			<i>Proteus mirabilis</i>		
	Growth control ^b	Water extract	Acetone extract	Growth control ^b	Water extract	Acetone extract
5000	1.047	0.714	0.511	0.285	0.276	0.121
2500		0.735	0.586		0.281	0.156
1250		0.735	0.611		0.281	0.193
625		0.744	0.609		0.298	0.205
312.5		0.865	0.703		0.301	0.215
156.25		0.755	0.838		0.348	0.251
78.12		1.016	0.925		0.362	0.258
39.06		1.163	1.235		0.493	0.312

^a Data represented absorbance at 630 nm.

^b Data shown is the mean value of the eight repetitions.

greater effect than water on the inhibition of biofilm formation of *P. aeruginosa* and *P. mirabilis*. In fact, the water extract showed no effect on the tested concentrations.

The acetone extract was effective at a concentration of 4469.5 $\mu\text{g/mL}$ for *P. aeruginosa* and 4315 $\mu\text{g/mL}$ for *P. mirabilis*. To the best of the authors' knowledge, the antibiofilm activity of water and acetone *A. eupatoria* extracts was examined for the first time.

4. Discussion

Because of its widespread use in folk medicine, agrimony provokes a great scientific interest. Many *A. eupatoria* phytochemical composition assays have focused on alcoholic and water–alcoholic extracts. In this study, chemical composition, antioxidant activity, and antibacterial properties of the acetone and diethyl ether extracts have been analyzed for the first time. In addition, the *in vitro* activity of water and acetone *A. eupatoria* extracts on biofilm formation was examined for the first time.

According to previous studies, *A. eupatoria* is very rich in secondary metabolites, and was reported to contain 3–11% of tannin and about 1.9% of flavonoids, phenolic acids, and triterpenoids [3–7]. Therefore, in this paper, we carried out a phytochemical analysis of the tested plant extracts. The highest concentrations of secondary metabolites were in the acetone extract, and the lowest was in the diethyl ether extract. Shabana et al [31] reported that the major compounds in the water–alcoholic extract were flavonoids (0.33%),

tannins (10.08%), and phenolic acids [2.26% luteolin 7-O-sophoroside, luteolin 7-O (6''-acetyl glucoside), acacetin 7-O-glucoside, luteolin 7-O-glucoside and apigenin 7-O-glucoside, protocatechuic acid, vanillic acids, and *p*-hydroxybenzoic acid]. The polyphenolic profile of plant extracts was characterized mainly by the high-performance liquid chromatography (HPLC) method. Dulger and Gonuz [32] isolated flavonoids, tannins, and terpenoids from ground parts of this plant. Kubinova et al [33] reported a high percentage of quercetin and apigenin glycoside in the HPLC profile of species from the *Agrimonia* genus, with the highest concentration of flavonoids being detected in the methanol extract of *A. eupatoria*. Our study confirmed that *A. eupatoria* is very rich in secondary metabolites.

4.1. Antioxidant activity

The high correlation between polyphenolic content and total antioxidant activity has been demonstrated in water–alcoholic agrimony extracts [14]. Ivanova et al [11] indicated that the antioxidant activity of *A. eupatoria* might be attributed to the chemical structure of polyphenols and/or to the result of activation of endogenous antioxidant defense systems. In this paper, the antioxidant activity of acetone, diethyl ether, ethanol, and water extracts of *A. eupatoria* was investigated and the results were compared with vitamin C. The higher concentrations of acetone extract and vitamin C operated in a similar way. All the tested extracts and the control substance showed a very strong antioxidant activity, and thus, this study further confirms the high antioxidant activity of agrimony.

In this study, the reducing power of all four tested extracts was examined for the first time, and they showed moderate reducing power compared with the positive control. In this case, the acetone extract was found to be the most active.

4.2. Antimicrobial and antifungal activities

Extracts of *A. eupatoria* possess powerful antibacterial activity. Dulger and Gonuz [32], using the disk-diffusion method, investigated the antimicrobial activity of the ethanol extract of some plant species, including *A. eupatoria*. Their results showed that *A. eupatoria* extracts exhibited antimicrobial activity on all kinds of microorganism strains studied, and the inhibition zone

Table 7 – Biofilm inhibitory concentration of water and acetone extracts of *Agrimonia eupatoria*.

Species	Water extract BIC ₅₀ ^a	Acetone extract BIC ₅₀	Tetracycline BIC ₅₀
<i>Pseudomonas aeruginosa</i>	>5000	4469.50	1522.50
<i>Proteus mirabilis</i>	>5000	4315	Not determined

BIC₅₀ = biofilm inhibitory concentration required to reduce biofilm coverage by 50%.

^a Data given as $\mu\text{g/mL}$.

diameters were 8–16 mm. In addition, *S. aureus*, *Listeria monocytogenes*, and *Micrococcus luteus* were found to be the most sensitive. Compared with *S. aureus*, the extract had greater effect than conventional antibiotics, except for ofloxacin and tetracycline. In the case of *L. monocytogenes*, the extract was more effective than the antibiotic ampicillin. In our research, the sensitivity of *S. aureus* was noticed in all tested extracts. Ghaima [34] studied the antibacterial effect of the water and ethanol *A. eupatoria* extracts on the strains of some pathogenic bacteria (*S. aureus*, *P. aeruginosa*, and *E. coli*). The results showed that the ethanol extract was more effective than the water extract, and this result was confirmed in our study. Cwikla et al [35] examined the effects of water–ethanol extract and essential oils of some plant species to determine their influence on *Helicobacter pylori* using the microdilution method and the results were compared with the inhibitory action of antibiotics. They concluded that the extracts of *A. eupatoria*, *Hydrastis canadensis*, *Filipendula ulmaria*, and *Salvia officinalis* were the most active in inhibiting the growth of *H. pylori*. Petkov [36] has been reported antibacterial activity for agrimony extracts against *S. aureus* and α -hemolytic streptococci.

In our research, the effect of water, acetone, diethyl ether, and ethanol extracts of *A. eupatoria* on fungi was closely examined for the first time and the results were compared with the effect of antifungal drugs. Compared with the positive controls, it could be concluded that all extracts exhibited low antifungal effect. According to Soliman and Badeaa [37], extracts of *A. eupatoria* leaves belong to the category of plant with low antiaflatoxin effect.

4.3. Antibiofilm activity

Bacterial biofilms are structures consisting of extracellular polymeric substances and single or multiple species of bacteria that form clusters and adhere to surfaces [38]. Biofilms are a self-protection growth pattern of bacteria, which are different from planktonic cells. They have been of considerable interest in food hygiene because biofilms may contain spoilage and reduce the risk of contamination by pathogenic bacteria, which increases postprocessing contamination and risk to public health [39]. The mechanisms of the effect of extracts on biofilm are different among plants. For example, curcumin (the main active compound of *Curcuma longa*), prevents the adherence of bacterial cells in the biofilm, making them more sensitive to antimicrobial agents rather than in biofilm state [40]. The effect of various plant extracts on the inhibition of biofilm formation has been documented for *Melilotus albus*, *Dorycnium herbaceum*, *Calendula officinalis*, and *Nigella sativa* [28,41,42].

In this study, for the first time, the antibiofilm activity of the acetone and water extracts of *A. eupatoria* was investigated on two bacteria, namely, *P. aeruginosa* and *P. mirabilis*, which have demonstrated the ability to form biofilm. We also investigated the effect of these extracts on inhibition on biofilm formation. The acetone plant extract was found to be very effective in inhibiting the bacterial biofilm formation. The antibiofilm activity could be related to the higher proportion of tannins and flavonoids in the plant [43]. This result is confirmed in our study, because the acetone extract showed the highest effect on the inhibition on biofilm formation, and

it also contained the higher proportion of tannins and flavonoids than the other tested extracts of *A. eupatoria*.

In this paper, it was shown that the extracts of *A. eupatoria* are a good source of bioactive compounds, what can be used in the food and pharmaceutical industries. Our study demonstrates the high phenolic content and antioxidant potential of *A. eupatoria* extracts and the study results may help in the development of beverages that could protect against free radical damage or safe food products and additives with appropriate antioxidant properties. Further studies must be carried out to include isolates of active compounds, elucidate the structures, toxicity testing, and also to evaluate the effects of plant extracts on the inhibition of biofilm formation.

4.4. Conclusion

Based on the study of acetone, diethyl ether, ethanol and water extracts of *A. eupatoria*, it could be concluded that all these extracts, especially acetone, are an important source of biologically active compounds. The study results also confirm the antioxidant and antibacterial properties of *A. eupatoria*; besides, the inhibition on biofilm formation by the acetone extract was significant. Additional effects of *A. eupatoria* extracts and their fine mechanisms of action are to be revealed by future studies.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

The authors are grateful to Professor Dr Marina Topuzović for identification and classification of the plant material. This investigation was supported by the Ministry of Education and Science of the Republic of Serbia (Grant Nos. 41010 and 173032).

REFERENCES

- [1] Josifovic M, Stjepanovic L, Jankovic MM, Gajic M, Kojic M, Diklic H. Flora of SR Serbia. Belgrade, Serbia: Serbian Academy of Sciences and Arts; 1972.
- [2] Saric M. Medicinal plants of SR Serbia. Belgrade, Serbia: Serbian Academy of Sciences and Arts; 1989.
- [3] Granica S, Krupa K, Klebewska A, Kiss KA. Development and validation of HPLC-DAD-CAD-MS(3) method for qualitative and quantitative standardization of polyphenols in *Agrimonia eupatoria* herba (Ph. Eur). *J Pharm Biomed Anal* 2013;86:112–22.
- [4] Senda J, Zieba J. Flavonoids from *Agrimonia eupatoria* L. *Diss Pharm Pharmacol* 1972;24:79–83.
- [5] Billa AR, Palme E, Catalano S, Pistelli L, Morelli I. Constituents and biological assay of *Agrimonia eupatoria*. *Fitoterapia* 1993;64:549–50.
- [6] Billa AR, Palme E, Catalano S, Pistelli L, Morelli I. A flavonol glycoside from *Agrimonia eupatoria*. *Phytochemistry* 1993;32:1078–9.

- [7] Feng XL, He YB, Liang YZ, Wang YI, Huang LF, Xie JW. Comparative analysis of the volatile components of *Agrimonia eupatoria* from leaves and roots by gas chromatography-mass spectrometry and multivariate curve resolution. *J Anal Methods Chem* 2013;2013:1–9.
- [8] Gray AM, Flatt PR. Actions of the traditional anti-diabetic plant, *Agrimonia eupatoria* (agrimony): effects on hyperglycaemia, cellular glucose metabolism and insulin secretion. *Br J Nutr* 1998;80:109–14.
- [9] Bratoeva K, Bekyarova G, Kiselova Y, Ivanova D. Effect of Bulgarian herb extracts of polyphenols on metabolic disorders—induced by high fructose diet. *Trakia J Sci* 2010;8:56–60.
- [10] Lee KY, Hwang L, Jeong EJ, Kim SH, Kim YC, Sung SH. Effect of neuroprotective flavonoids of *Agrimonia eupatoria* on glutamate-induced oxidative injury to HT22 hippocampal cells. *Biosci Biotechnol Biochem* 2010;74:1704–6.
- [11] Ivanova D, Kiselova-Kaneva Y, Vankova D, Tasinov O. Protection by *Agrimonia eupatoria* extracts of oxidant-challenged 3T3-L1 pre-adipocyte cells. *Obes Rev* 2011;12:176–7.
- [12] Yoon SJ, Koh EJ, Kim CS, Zee OP, Kwak JH, Jeong WJ, Kim JH, Lee SM. *Agrimonia eupatoria* protects against chronic ethanol-induced liver injury in rats. *Food Chem Toxicol* 2012;50:2335–41.
- [13] Ad'hiah AL, Al-Bederi ONH, Al-Sammarraie KW. Cytotoxic effects of *Agrimonia eupatoria* L. against cancer cell lines in vitro. *J Assoc Arab Univ Basic Appl Sci* 2013;14:87–92.
- [14] Kiselova Y, Galunska B, Ivanova D, Yankova T. Total antioxidant capacity and polyphenol content correlation in aqueous-alcoholic plant extracts used in phytotherapy. *Scr Sci Med* 2004;36:11–3.
- [15] Gão M, Pestana D, Faria A, Guimarães J, Pintado M, Calhau C, Azevedo I, Malcata F. Effects of extracts of selected medicinal plants upon hepatic oxidative stress. *J Med Food* 2010;13:131–6.
- [16] Ivanova D, Vankova D, Nashar M. *Agrimonia eupatoria* tea consumption in relation to markers of inflammation, oxidative status and lipid metabolism in healthy subjects. *Arch Physiol Biochem* 2013;119:32–7.
- [17] Kwon DH, Kwon HY, Kim HJ, Chang EJ, Kim MB, Yoon SK, Song EY, Yoon DY, Lee YH, Choi IS, Choi YK. Inhibition of hepatitis B virus by an aqueous extract of *Agrimonia eupatoria* L. *Phytother Res* 2005;4:355–8.
- [18] Chakarski I. Clinical study of a herb combination consisting of *Agrimonia eupatoria*, *Hipericum perforatum*, *Plantago major*, *Mentha piperita*, *Matricaria chamomila* for the treatment of patients with chronic gastroduodenitis. *Probl Vatr Med* 1982;10:78–84.
- [19] Wootton-Beard PC, Moran A, Ryan L. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Res Int* 2011;44:217–24.
- [20] Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin JC, Bailleul F, Trotin F. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *J Ethnopharmacol* 2000;72:35–42.
- [21] Makkar HPS, Blummel M, Borowy NK, Becker K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *J Sci Food Agric* 1993;61:161–5.
- [22] Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 1986;25:223–30.
- [23] Takao T, Kitatani F, Watanabe N, Yagi A, Sakata K. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Biosci Biotechnol Biochem* 1994;58:1780–3.
- [24] Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Nutr* 1986;44:307–15.
- [25] Andrews JM. BSAC standardized disc susceptibility testing method (version 4). *J Antimicrob Chemother* 2005;56:60–76.
- [26] Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods* 2007;42:321–4.
- [27] O'Toole G, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 1998;28:449–61.
- [28] Chaieb K, Kouidhi B, Jrah H, Mahdouani K, Bakhrouf A. Antibacterial activity of thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation. *BMC Complement Altern Med* 2011;11:29.
- [29] Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? *J Ethnopharmacol* 1988;60:1–8.
- [30] Tajkarimi MM, Ibrahim SA, Cliver DO. Antimicrobial herb and spice compounds in food. *Food Control* 2010;21:1199–218.
- [31] Shabana M, Weglarz Z, Geszprych A. Phenolic constituents of agrimony (*Agrimonia eupatoria* L.) herb. *Herba Pol* 2003;49:24–8.
- [32] Dulger B, Gonuz A. Antimicrobial activity of some Turkish medicinal plants. *Pak J Biol Sci* 2004;7:1559–62.
- [33] Kubinova R, Jankovska D, Bauerova V. Antioxidant and α -glucosidase inhibition activities and polyphenol content of five species of *Agrimonia* genus. *Acta Fytotech Zootech* 2012;2:4–38.
- [34] Ghaima KK. Antibacterial and wound healing activity of some *Agrimonia eupatoria* extracts. *Baghdad Sci J* 2013;10:152–60.
- [35] Cwikla C, Schmidt K, Matthias A, Bone KM, Lehmann R, Tiralongo E. Investigations into the antibacterial activities of phytotherapeutics against *Helicobacter pylori* and *Campylobacter jejuni*. *Phytother Res* 2010;24:649–56.
- [36] Petkov V. Bulgarian traditional medicine: a source of ideas for phytopharmacological investigations. *J Ethnopharmacol* 1986;15:121–32.
- [37] Soliman KM, Badaea RI. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food Chem Toxicol* 2002;40:1669–75.
- [38] Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 2000;64:847–67.
- [39] Xianming S, Xinna Z. Biofilm formation and food safety in food industries. *Trends Food Sci Technol* 2009;20:407–13.
- [40] Man TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 2001;9:34–9.
- [41] Stefanovic O, Tesic J, Comic LJ. *Melilotus albus* and *Dorycnium herbaceum* extracts as source of phenolic compounds and their antimicrobial, antibiofilm, and antioxidant potentials. *J Food Drug Anal* 2015;23:417–24.
- [42] Ghaima KK, Rasheed SF, Ahmed EF. Antibiofilm, antibacterial and antioxidant activities of water extract of *Calendula officinalis* flowers. *Int J Pharma Bio Sci* 2013;4:465–70.
- [43] Siqueira CF, Cabral DLV, Sobrinho SP, Amorim ELC, de Melo JG, Araújo AS, Albuquerque UP. Levels of tannins and flavonoids in medicinal plants: evaluating bioprospecting strategies. *Evid Based Complement Alternat Med* 2012;2012:1–7.