Screening for antioxidant, antifungal, and antitumor activities of aqueous extracts of chamomile (*Matricaria chamomilla*)

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Purpose
The aim of this study was to investigate the optimum conditions to isolate aqueous extracts of chamomile seeds at different pHs and modify chemically by means of sulfation. In this work, the extracts of chamomile seeds were obtained by means of water extraction (80°C) at three different concentrations (acidic, neutral, and alkaline conditions), followed by sulfation with chlorosulfonic acid.

Materials and methods
In all obtained extracts and sulfated derivatives, the content of total phenols and flavonoids was determined. The antioxidant activities of the extracts were measured using the diphenyl-1-picrylhydrazyl radical scavenging activity method and displayed, in general, the moderate activity for all studied extracts. The agar diffusion method was utilized to screen the antifungal effects of both extracts and their sulfated derivatives. The antitumor effect of extracts at different concentrations (300, 600, and 900 μg/ml) was measured microscopically using the cell cancer viability test.

Results
The aqueous extracts and their corresponding sulfates produced the same activity against two strains of *Aspergillus niger* and *Penicillium citrinum* at the concentration of 40 μg/disc as compared with the reference commercial fungicidal griseofulvin. The activity of all extracts revealed slight inhibition of Ehrlich ascites carcinoma cell line growth.

Conclusion
Water extracts of chamomile plant seeds obtained at different pH conditions demonstrated moderate antioxidant, antifungal, and antitumor activities. Further studies are needed to increase the efficiency of extraction methods to increase phenolic and flavonoid proportions in extracts.

Keywords:
antifungal, antioxidant, antitumor, aqueous extract, chamomile, sulfated extracts

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Introduction

All over the world, there is an increasing interest in medicinal plants. Researchers, as well as the public, in general, recognize that natural products, predominantly those derived from plants, exhibit benefits for human health [1]. For centuries, traditional medicine has provided a crucial health support for millions of people around the globe. In some countries, less than 20% of population has access to basic generic medicines or healthcare products [2].

The use of natural products as biocompatible and nontoxic drugs without side effects in medicine is highly favored compared with chemical and synthetic drugs. In recent years, there has been a growing interest in natural extracts exhibiting biological and medicinal properties that are supplied to humans and animals as food components or as specific pharmaceutics. Medicinal plants are considered as the resources of promising drugs for many diseases. However, the biological and pharmacological properties of many plants are still unknown. Plant extracts are the sources of naturally occurring antioxidants and antitumor agents [3].

Chamomile (*Chamomilla recutita*) belongs to the Compositae family and grows in Europe, North East Asia, and North and South America. Some countries that produce chamomile for the international market are as follows: Argentina, Egypt, Bulgaria, Hungary, Spain, Czech Republic, Germany, Brazil, Chile, and Peru [4]. Chamomile has been one of the most widely used and well-documented medicinal plants for centuries. It has been generally used for the antioxidant, antimicrobial, antiplatelet, anti-inflammatory, and even cancer-suppressive activities of chamomile constituents [5,6]. In addition, chamomile extracts are

widely used in the cosmetology industry to impart flavor to a number of personal care products. The therapeutic activity of chamomile belongs to different effective substances such as phenolics and flavonoids apigenin, quercetin, patuletin, luteolin, and their glucosides.

Many authors have studied the antioxidant potential of chamomile extracts and compared with the antioxidant capacity of other herbal sources. Dragland *et al.* [7] reported that the biopotential of chamomile extracts was higher than that exhibited by coriander, but lower in comparison with peppermint and oregano. Using two different assays, Lee and Shibamoto [8] evaluated the antioxidant activities of thyme and basil and chamomile volatile extracts compared with those of the known antioxidants butylated hydroxytoluene and R-tocopherol.

In addition, previous investigations proved the antimicrobial activity of chamomile [9–11]. Extracts of this plant have been shown to be inhibitory against a wide range of microbial strains, depending on their concentration, method of testing, and composition. Bearing in mind that phenolic compounds are known potent antimicrobial agents [12–16], the high antimicrobial activity of chamomile flower extracts could be linked to the high phenol concentration.

The main purpose of this work was to study the optimum conditions to isolate aqueous extracts of chamomile seeds at different pHs and modify chemically by means of sulfation. The antimicrobial, antioxidation, and antitumor potentials of aqueous extracts and their corresponding derivatives were evaluated.

Materials and methods

Plant materials

Chamomile seeds (*Matricaria chamomilla*) were purchased and identified by the Central Administration of Horticulture and Agricultural crops, Ministry of Agriculture and Land Reclamation (Egypt), and milled before extraction.

Chemicals

Hemoclar (pentosan sulfuric polyester) was purchased from the Nile Co. Pharmaceuticals, Cairo, Egypt. Heparin was purchased from Sigma Chemical Co. Plasma was purchased from the Egyptian Organization for Biological Products and Vaccine Production. Control antibiotic discs were purchased from Oxoid.

Analytical methods

For each studied variety, the chemical properties of moisture, ash, crude protein, and crude lipids were determined according to the method of Association of Official Analytical Chemistry (AOAC) [17]. Total carbohydrates were determined after complete acid hydrolysis [18]. The resulted acid hydrolysates were examined with a PC using *n*-BuOH-MeCO-H_2O (4 : 5 : 1) [19] and aniline phthalate [20] as spraying reagents. Quantitative determination of the separated sugars was carried out according to the method of Wilson [21]. Total nitrogen of the investigated samples (0.3 g) was determined by adopting the usual micro-Kjeldahl's method [17]. The crude protein was calculated by multiplying the total nitrogen by 6.25 [22].

Preparation of crude aqueous extracts

Extraction of plant was singly carried out with 0.5 N HCl, water, and 0.5 N NaOH. Briefly, chamomile sample (5 g) was extracted with 200 ml of extracting solvent at 80°C for 3 h. After filtration, the extracts were neutralized and dialyzed against distilled water for 48 h, dried, and weighed. The chemical characterization of the extracts was achieved by determining their total carbohydrates and the monosaccharide constituents of the extracts hydrolyzed. The methods used for these analyses were previously mentioned [17–20]. Soluble protein was estimated using the Lowry method [23].

Preparation of sulfated extracts

By adopting the method reported by Yang *et al.* [24] with some modification, the sulfation of the water-free extract was performed as follows: 0.1 g of water-free extract was suspended in 0.5 ml dry formamide, and the mixture was stirred at room temperature for 24 h to disperse it into the solvent. A sulfating agent was prepared by adding 1 ml of $HClSO_3$ dropwise in 4 ml of formamide under cooling in an ice-water bath and then added to the extract. The reaction was cooled in ice, neutralized with 30% NaOH solution, and dialyzed against running water for 48 h and then lyophilized.

Microorganisms and media

The antifungal activity of aqueous extracts and their sulfated derivatives were individually tested against a panel of two fungi (*Penicillium citrinum* and *Aspergillus niger*). The strains were grown on Sabouraud dextrose agar.

Preparation of the inoculums

Culture media

Sabouraud dextrose agar (g/l) comprised the following: peptone, 10.0 g; glucose, 20.0 g; and agar-agar, 17.0 g, with chloramphenicol 0.5 g for yeasts and molds. The inoculum used for all assays reached the microbial density of the order of 10^8–10^9 spores/ml.

Chemicals, solvents, and reagents

Diphenyl-1-picrylhydrazyl (DPPH), trypan blue, gallic acid, quercetin, and Folin–Ciocalteu reagents were obtained from Sigma Chemical Co. All other solvents and chemicals were of analytical grade.

Animal and tumor

Female Swiss albino mice (8–10 weeks) weighing 22–25 g were used. The animals were housed in polycarbonate cages in a room with a 12 h day–night cycle, temperature of 25°C, humidity of 45–65%, and were fed a balanced commercial diet and water. Ehrlich ascites carcinoma cells, derived from a spontaneous murine mammary adenocarcinoma, were maintained in the ascites form by peritoneal transplantation of 2×10^6 cells. EAT cells were counted in a hemocytometer slide. The cells were found to be more than 99% viable using the trypan blue dye exclusion method.

Chemical characterization of crude aqueous extracts

The carbohydrate content was analyzed using the phenol–H_2SO_4 method [18] without previous hydrolysis of the polysaccharide. Total protein was estimated using the method of Lowry *et al.* [23]. The sugar composition was determined after complete hydrolysis with H_2SO_4 (2 mol/l) at 100°C for 8 h, neutralized with $BaCO_3$, and then centrifuged, filtered, neutralized with Dowex 50 resin (H^+ form), and concentrated. The hydrolyzed products were spotted in Whatman no.1 paper and subjected to chromatography [21] in butanol : acetone : water (4 : 5 : 1 v/v) for 24 h. The chromatogram was visualized by spraying with aniline phthalate [20].

Determination of total phenolics

The total phenols in aqueous extracts of powdered chamomile seeds were estimated according to the method described by Makkar *et al.* [25]. A volume of 1 ml of the extract was taken in a test tube, and then 0.5 ml of Folin–Ciocalteu reagent (1 : 1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. After 1 h of incubation at room temperature, the absorbance was measured at 725 nm and compared with a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid equivalent/g extract), and the values are presented as means of triplicate.

Determination of total flavonoids

Total flavonoid content was determined spectrophotometrically using the method of Ordonez *et al.* [26] based on the formation of a complex flavonoid-aluminum. An aliquot (0.5 ml) of aqueous extract was mixed with $AlCl_3$ solution (2%, 0.5 ml). Thereafter, the mixture was properly mixed and allowed to stand for 30 min at room temperature. The intensity of color was measured at 420 nm after filtration, if necessary. Total flavonoid content was calculated as quercetin equivalent from a calibration curve and the values were presented as means of triplet analyses.

Antioxidant activity (diphenyl-1-picrylhydrazyl assay)

The free radical scavenging activity using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) reagent was determined according to Brand-Williams *et al.* [27]. The samples (10 mg) were extracted with 80% aqueous methanol. To 0.75 ml of the extract sample, 1.5 ml of freshly prepared DPPH solution (prepared with 20 mg of DPPH/l of methanol) was added and stirred. The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control. The percentage of DPPH scavenging activity is expressed by the following formula: antioxidant activity%=[(control absorbance−sample absorbance/control absorbance×100)]. The values were presented as means of triplet analyses.

Antifungal screening

The paper disc diffusion technique [28] was used to test the microbial activity of aqueous extracts of chamomile seeds as follows: the agar plate containing the appropriate medium was spread with the inoculums containing 10^{10} CFU/ml. The filter paper disc (5 mm in diameter, Whatman no. 3) was absorbed with 0.025 ml of extract (1.6 mg/ml) and then placed onto agar plates. After incubation at 28°C and 7 days at 28°C, the diameters of inhibition zones were measured in mm.

Positive activity was defined as an inhibition zone of over 7 mm surrounding a disc [29]. Griseofulvin (20 μg/disc) was used as positive control.

Trypan blue exclusion test

To detect the cell viability, the trypan blue exclusion test was performed; the suspension of the tumor cells was attained from peritoneal cavities of tumor-bearing mice and then diluted with PBS (pH=7) so that the final preparation comprised 2.5×10^5 cells/0.1 ml. Briefly, in a set of sterile test tubes, aliquots (0.1 ml/tube) of the cell preparation were distributed, followed by addition of aliquots (0.8 ml/tube) of PBS. The investigated samples (dissolved in PBS) were then applied to the tubes in aliquots (0.1 ml/tube) at different concentration of the dry samples. The tubes were incubated at 37°C for 2 h under 5% CO_2. Subsequently, the tubes were centrifuged at 1000 rpm for 5 min and the separated cells were suspended in saline. For each examined tube

and control, a new clean, dry small test tube was used and 0.1 ml of cell suspension, 0.8 ml of saline, and 0.1 ml of trypan blue were added and mixed, and then the number of living cells was calculated using a hemocytometer slide. Viable cells appeared as unstained bodies, whereas nonviable cells stained blue [30].

Statistical analysis

Data were statistically analyzed using SPSS, version 10.00, for Windows (SPSS Inc., Chicago, Illinois, USA). Data were presented as mean±SD.

Results and discussion

Chemical composition

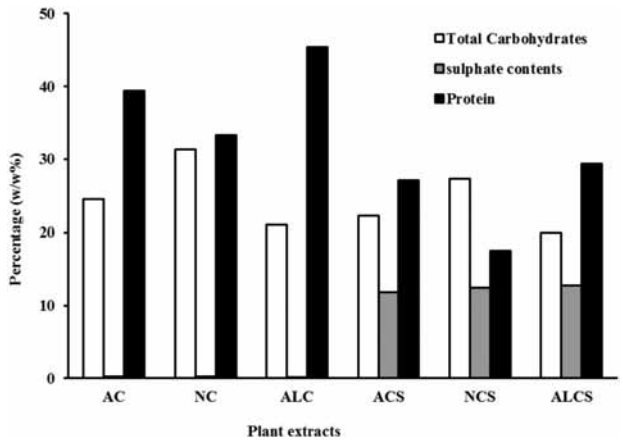
The aqueous extraction was performed under different pH conditions (pH=3, 7 and 12) at 80°C to extract hydrophilic water-soluble oligopolymers and biopolymers such as polysaccharides, phenolic compounds, and flavonoid compounds. Both kinds of high and low molecular weight hydrophilic compounds play important roles in the medicinal functions of chamomile seeds. To increase the biological potential of the aqueous extracts, chemical sulfation was performed as one of the most important chemical modifications. The chemical composition of aqueous extracts and their corresponding sulfated derivatives were determined. The results of total carbohydrates, water-soluble proteins, and sulfate contents for the three extracts and their corresponding sulfated derivatives are shown in Fig. 1.

On the basis of the data presented in Fig. 1, NC and ALC extracts showed the highest total carbohydrates and protein concentrations (31.3 and 45.4%, respectively). In the meanwhile, lowest concentration of total carbohydrates and water-soluble protein was recorded in ALCS and NCS (20 and 17.5%, respectively). The sulfate functional group is not present in all extracts.

In continuous vein of this study, the total carbohydrates in extracts exhibited the second major part after protein. As the carbohydrate contents varied from 20 to 30%. The carbohydrate contents represented the high yield (31.3%) in NC extract. In contrast, the ALC extract comprised a low yield of total carbohydrates (21%). The sulfation of three aqueous extracts was achieved and the sulfate content was around 10% for all three modified derivatives.

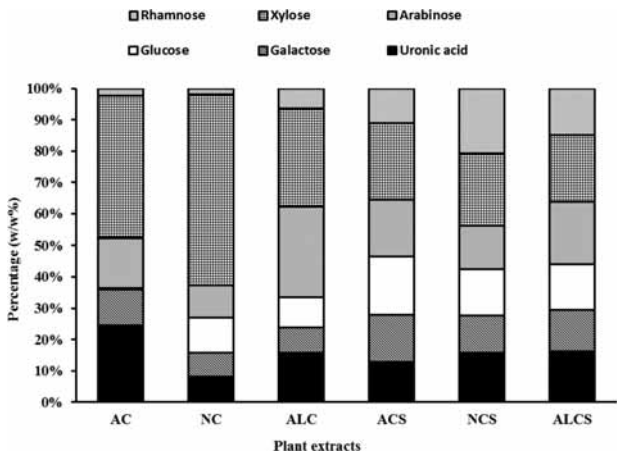
Complete acid hydrolysis of the three aqueous extracts and their corresponding sulfated derivatives, followed by quantitative determination of the monosaccharides resulted from carbohydrate hydrolyzates, indicated the presence of xylose, arabinose, and uronic acids as major components, in addition to lower proportions of glucose, galactose, and rhamnose (Fig. 2). In general, xylose represents the major monosaccharide component in all extracts and corresponding derivatives as the highest values of xylose in NC extract was 60.9% and the lowest value in ALCS was 21.3%. Next to xylose, the highest value of arabinose was recorded in ALC (28.7%) and the lowest value in NC (10.3%). Furthermore, the highest value of uronic acid was represented in AC extract (23.6%) and the lowest value in NC extract (8.1%). With respect to rhamnose, the highest value was found in NCS extract (20.7%) and the lowest value in AC extract (2.3%). In contrast, the values of glucose and galactose monosaccharides increased with decreasing xylose and arabinose during

Figure 1



Chemical composition of aqueous extract of chamomile and sulfated derivatives.

Figure 2



Relative constituents of monosaccharides of aqueous extracts and their sulfated derivatives.

sulfation. These findings indicated that the carbohydrate contents of chamomile extracts hydrolyzed during sulfation. Xylose and arabinose, pentose-monosaccharide family, are highly labile during sulfation process compared with glucose, galactose, rhamnose, and uronic acids.

Phenolic content

The structural variety of phenolics offered a significant challenge for developing a general methodology that is suitable for extraction of all phenolics. The problem of developing a satisfactory extraction procedure is further complicated as phenolics are not equally distributed in plants at the tissue, cellular, and subcellular levels. In addition, these compounds can be found in free, conjugated, and polymeric forms, or may coexist as complexes with carbohydrate, protein, or other plant components. All of the above factors directly impact the solubility of phenolics in different solvents. In this study, the extraction of phenolics was carried out in aqueous phase at three different pH conditions to extract free and conjugated phenolic compounds with hydrophilic characters.

The results obtained in the present study revealed that the total phenolic contents in the aqueous extracts of the seeds of chamomile seeds and their sulfated derivatives were considerably significant (Fig. 3). Polyphenolic compounds are known to have antioxidant activity, and it is likely that the activity of the extracts is due to these compounds. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [31].

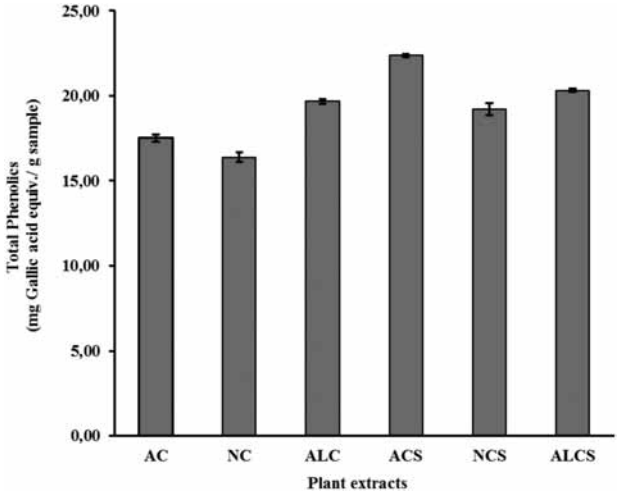
The total phenolic concentrations of the aqueous extracts and their sulfated derivatives are shown in Fig. 3. Phenolic compounds in the three aforementioned extracts ranged from 16.4 (NC) to 19.7 (ALC) mg gallic quiv/g of dry extract, whereas the phenolics in the corresponded sulfated derivatives are a little bit higher compared with the native extract. The total phenolics in three sulfated derivatives were from 19.2 (NCS) to 22.4 (ACS) mg gallic quiv/g of dry extract. These findings might be due to the hydrolysis of conjugated phenolics and be easily extracted with aqueous methanol used for screening of total phenolics.

As regards total flavonoids, Fig. 4 shows the flavonoid concentrations in the investigated plant extracts and their corresponding sulfated derivatives. Flavonoids have been reported to have useful properties, including anti-inflammatory activity, enzyme inhibition, and antimicrobial activity [32,33]. The highest amount of flavonoids (1.8 mg/g) was detected in AC extract and the lowest amount (1.1 mg/g) was detected in NC extract.

Antioxidant activity (diphenyl-1-picrylhydrazyl radical scavenging activity)

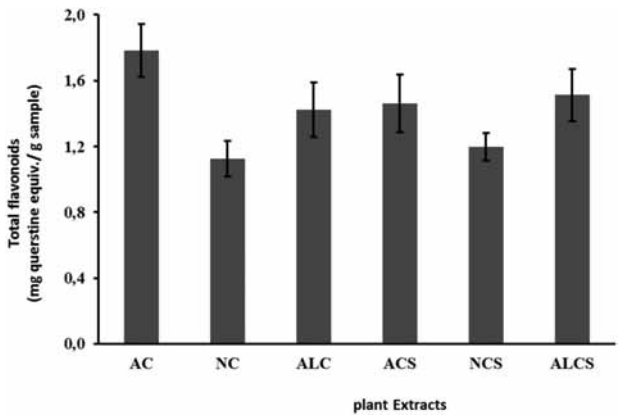
DPPH radical scavenging activity of chamomile extracts (AC, NC, and ALC) and their sulfated derivatives (ACS, NCS, and ALCS) are shown in Fig. 5. In general, three different extracts and their sulfated derivatives have moderate antioxidant activities. At a concentration of 10 mg/ml, the scavenging activity of NCS reached 26.5%, with 25.3% for AC, 24.7% for ACS, 24.3% for NC,

Figure 3



Total phenolics of aqueous extracts of chamomile and their sulfated derivatives.

Figure 4



Total flavonoids of aqueous extracts of chamomile and their sulfated derivatives.

23.3% for ALC, and 22.3% for ALCS. The effect of antioxidants on DPPH is thought to be due to their hydrogen-donating ability. The study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The radical scavenging activity of the extracts could be related to the nature of phenolics and their hydrogen-donating ability [34].

Antifungal activity

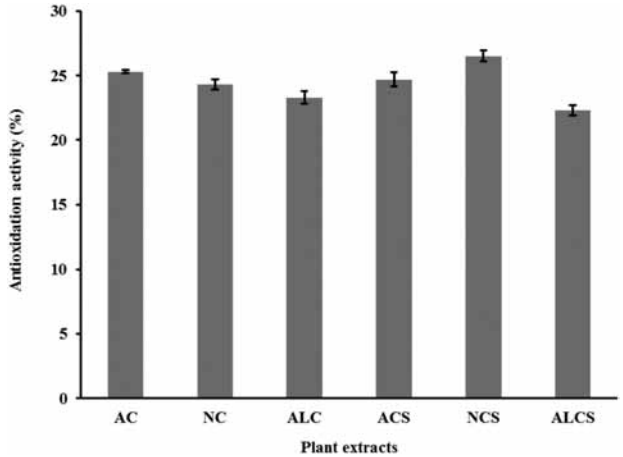
It has often been reported that water extract do not have biological activity [35]. However, in this study, water extracts of chamomile exhibited moderate antifungal activity against *P. citrinum* and *A. niger*. This justifies the use of water extracts in traditional medicine [36].

The antifungal activity *in vitro* of the aqueous extracts and sulfated derivatives were tested against *P. citrinum* and *A. niger* compared with the antifungal activity of griseofulvin, the most effective drug for the treatment of fungal diseases.

Table 1 Antifungal activity of aqueous extracts and their sulfated derivatives using disc diffusion assay

Extract	Inhibition zone (mm)	
	P. citrinum	*A. niger*
AC	12	10
NC	10	10
ALC	10	10
ACS	10	7
NCS	12	10
ALCS	12	10
Standard (griseofulvin)	16	18

Figure 5



Antioxidation activity of aqueous extracts of chamomile and their sulfated derivatives.

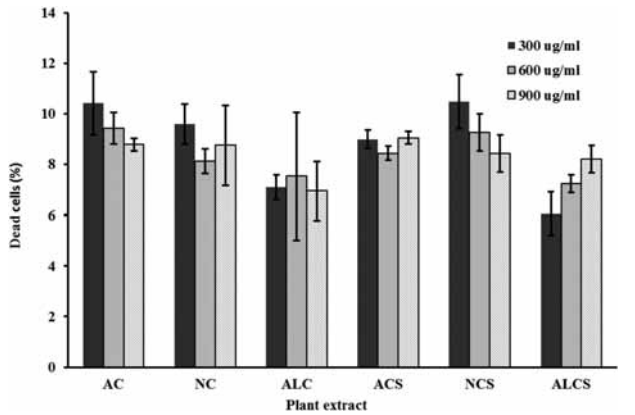
Our protocol was not designed to address the mode of action of chamomile flowers' extract, but its antimycotic and antibacterial properties have been reported already. As shown in Table 1, the three aqueous extracts had relative antifungal activity. In general, the six extracts from aqueous and corresponding sulfated derivatives produced the same activity against two strains of *A. niger* and *P. citrinum* at the concentration of 40 μg/disc as compared with the reference commercial fungicidal griseofulvin (Table 1). The diameter of the inhibition zones produced against two strains of *A. niger* and *P. citrinum* ranged from 10 to 12 mm for the aqueous extracts and 7 to 12 mm for sulfated derivatives.

Antitumor activity

The amount of research into the utility of chamomile continues to increase because of its reported biological activities, which appear to be related to its content of several classes of bioactive compounds. A part of our study aim was to demonstrate that chamomile plant extracts at different pH and their corresponding sulfated derivatives cause the suppression of cancer cell growth and thus causes apoptosis.

Figure 6 shows the effect of three aqueous extracts and their sulfated derivatives at three different concentrations (300, 600, and 900 μg/ml)) on the viability of EAC cells *in vitro*. It was observed that there was slight decrease in the viability with increasing extract concentration. In general, the results of the antitumor activity were rather disappointing. All three extracts and their sulfated derivatives showed moderate antitumor activity against the Ehrlich ascites carcinoma cells. In contrast, as has been previously reported [37],

Figure 6



In-vitro antitumor activities of aqueous extracts of chamomile and their sulfated derivatives.

in-vivo antitumor activity of aqueous and methanolic extracts showed a significant decrease in cell viability in various human cancer cell lines.

Conclusion

Water extracts of chamomile plant seeds obtained at different pH conditions demonstrated moderate antioxidant, antifungal, and antitumor activities. Further studies are needed to increase the efficiency of extraction methods to increase phenolic and flavonoid proportions in extracts.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Petronilho S, Maraschin M, Coimbra MA, Rocha M. *In vitro* and *in vivo* studies of natural products: a challenge for their valuation. The case study of chamomile (*Matricaria recutita*). Ind Crops Prod 2012; 40:1–12.

2 Hogerzei HV, Mirza Z. The world medicines situation (2011); access to essential medicines as part of the right to health, Geneva

3 Matić IZ, Juranić Z, Savikin K, Zdunić G, Nađvinski N, Gođevac D. Chamomile and marigold tea: chemical characterization and evaluation of anticancer activity (2012); Phytotherapy Research

4 Povh NP, Meireles MAA. Marques MOM supercritical CO_2 extraction of essential oil and oleoresin from chamomile [Matricaria recutita (L.) Rauschert]. Proceedings of 2nd International Meeting on High Pressure Chemical Engineering; 2001; (Anais em CDROM), Hamburgo.

5 Bhaskaran N, Shukla S, Srivastava JK, Gupta S. Chamomile: an anti-inflammatory agent inhibits inducible nitric oxidesynthase expression by blocking RelA/p65 activity. Int J Mol Med 2010; 26:935–940.

6 McKay DL, Blumberg JB. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.) Phytother Res 2006; 20 (7): 519–530.

7 Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several culinary and medicinal herbs are important sources of dietary antioxidants. J Nutr 2003; 133(5):1286–1290.

8 Lee KG, Shibamoto T. Determination of antioxidant potential of volatile extracts isolated from various herbs and spices. J Agric Food Chem 2002; 50(17):4947–4952.

9 Cvetanović A, Švarc-Gajić J, Mašković P, Savić S, Nikolić L. Antioxidant and biological activity of chamomile extracts obtained by different techniques: perspective of using superheated water for isolation of biologically active compounds. Ind Crops Prod 2015; 65:582–910.

10 Shikov AN, Pozharitskaya ON, Makarov VG, Kvetnaya AS. Antibacterial activity of *Chamomilla recutita* oil extract against *Helicobacter pylori*. Phytother Res 2008; 22(2):252–253.

11 Franke R, Schilcher H. Chamomile: Industrial Profiles, II Series: Medicinal and Aromatic Plants 2005; 42: CRC Press/Taylor & Francis Group, Boca Raton/Broken Sound Parkway

12 Pereira JA, Pereira AP, Ferreira IC, Valentão P, Andrade PB, Seabra R *et al.* Table olives from Portugal: phenolic compounds, antioxidant potential, and antimicrobial activity. J Agric Food Chem 2006; 54 (22):8425–8431.

13 Pereira AP, Ferreira IC, Marcelino F, Valentão P, Andrade PB, Seabra R, *et al.* Phenolic compounds and antimicrobial activity of olive (Olea europaea L. Cv. Cobrançosa) leaves. Molecules 2007; 12(5):1153–1162.

14 Pereira JA, Oliveira I, Sousa A, Valentaö P, Andrade PB, Ferreira ICFR, *et al.* Walnut (*Juglans regia L.*) leaves: phenolic compounds, antimicrobial activity and antioxidant potential of different cultivars. Food Chem Toxicol 2007b; 45:2287–2295.

15 Proestos C, Chorianopoulos N, Nychas GJ, Komaitis M. RP-HPLC analysis of the phenolic compounds of plant extracts. investigation of their antioxidant capacity and antimicrobial activity. J Agric Food Chem 2005; 53(4):1190–1195.

16 Rauha JP, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, *et al.* Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int J Food Microbiol 2000; 56(1):3–12.

17 Association of Official Analytical Chemistry (AOAC). Official methods of analysis. Arlington, VA, USA: Association of Official Analytical Chemistry; 1995:25–28.

18 Dubois M, Gilles KA, Hamiton JK, Rebers PA, Smith M. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28:350–356.

19 Jayme G, Knoll H. Paper chromatography of sugar mixtures up on glass-fiber paper. Angew Chem 1956; 68:243–246.

20 Partridge SM. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 1949; 164(4167):443.

21 Wilson CM. Quantitative determination of sugars on paper chromatograms. Anal Chem 1959; 31:1199–1201.

22 Conklin-Brittain NLES, Dierenfeld RW, Wrangham M, Norconk SC. Silver chemical protein analysis: a comparison of Kjeldahl crude protein and total ninhydrin protein from wild, tropical vegetation. J Chem Ecol 1999; 25:2601–2622.

23 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193(1):265–275.

24 Yang XB, Gao XD, Han F, Tan RX. Sulfation of a polysaccharide produced by a marine filamentous fungus Phoma herbarum YS4108 alters its antioxidant properties *in vitro*. Biochim Biophys Acta 2005; 1725(1):120–127.

25 Makkar HPS, Becker K, Abel H, Pawelzik E. Nutrient contents, rumen protein degradability and antinutritional factors in some colour and white- flowering cultivars of Vicia faba beans. J Sci Food Agric 1997; 75:511–520.

26 Ordonez AAL, Gomez JD, Vattuone MA, Lsla MI. Antioxidant activities of Sechium edule (Jacq.) swartz extracts. Food Chem 2006; 97:452–458.

27 Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensmittel Wissenschaften und Technologi 1995; 28:25–30.

28 Adwan G, Abu-Shanab B, Adwan K, Abu-Shanab F. Antibacterial effects of nutraceutical plants growing in palestine on pseudomonas aeruginosa. Turk J Biol 2006; 30:239–242.

29 Irkin R, Korukluoglu M. Effectiveness of Cymbopogon citratus L. essential oil to inhibit the growth of some filamentous fungi and yeasts. J Med Food 2009; 12(1):193–197.

30 El-Merzabani MM, El-Aaser AA, Attia MAM, El-Duweini AK, Ghazal AH. Screening system for Egyptian plants with potential antitumor activity. J Planta Medica 1979; 36:150–155.

31 Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem 2001; 49(11):5165–5170.

32 Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 1983; 32(7):1141–1148.

33 Harborne JB, Baxter H. The handbook of natural flavonoids. Chichester, United Kingdom: John Wiley and Sons; 1999. vols. 1 and 2

34 Shimada KK, Fujikawa KY, Nakamura T. Antioxidative properties of xanthone on autoxidation of soybean oil in cyclodextrin. J Agric Food Chem 1992; 40:945–948.

35 Luseba D, Elgorashi EE, Ntloedibe DT, Van Staden J. Antibacterial, antiinflammatory and mutagenic effects of some medicinal plants used in South Africa for treatment of wounds and retained placenta in livestock. South Afr J Bot 2007; 73:378–383.

36 Mulaudzi RB, Ndhlala AR, Finnie JF, Van Staden J. Antimicrobial, antiinflammatory and genotoxicity activity of Alepidea amatymbica and Alepidea natalensis (Apiaceae). South Afr J Bot 2009; 75:584–587.

37 Srivastava JK, Gupta S. Antiproliferative and apoptotic effects of chamomile extract in various human cancer cells. J Agric Food Chem 2007; 55:9470–9478.