Survey of Phytochemical Composition and Biological Effects of Three Extracts from a Wild Plant (Cotoneaster nummularia Fisch. et Mey.): A Potential Source for Functional Food Ingredients and Drug Formulations

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Abstract
This study was focused on the analysis of the phenolic content, antioxidant, antibacterial, anti-cholinesterase, anti-tyrosinase, anti-amylase and anti-glucosidase activity of three solvent extracts from Cotoneaster nummularia. Moreover, water extract was tested in terms of mutagenic/anti-mutagenic effects. The antioxidant activities of these extracts were evaluated by DPPH, ABTS, O₂-, metal chelating, phosphomolybdenum, β-carotene/linoleic acid, ferric and cupric reducing power assays. Enzyme inhibitory activities were also examined with colorimetric methods. Generally, methanol and water extracts exhibited excellent biological activities. These extracts were rich in phenolic and flavonoid content. Furthermore, Cotoneaster extracts indicated appreciable antibacterial properties against human pathogen strains. HPLC analysis showed that ferulic acid, chlorogenic acid, (-) -epicatechin and (+)-catechin were the major phenolics in extracts tested. These data offer that these extracts from C. nummularia may be considered as a potential source of biological agents for developing functional foods or drug formulations.

Introduction
In the past decade, an increasing interest in the use of natural bioactive compounds origin from plant for scientific research as well as different purposes such as pharmaceutical and food industries [1]. For example, plant antioxidants are very significant for aforementioned areas, because many synthetic antioxidants (butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG)) have possible activity as promoters of carcinogenesis [2]. In addition, natural products are an important group of preventive agents for the treatment of global health problems such as Alzheimer’s diseases (AD), diabetes mellitus (DM). Inhibition of the key enzymes became a widely used treatment strategy in the pathogenesis of AD and DM. To this end, many enzyme inhibitors are produced synthetically for these diseases. For instance, Galatamine, tacrine are employed to be cholinesterase inhibitors to treat AD. Likewise, acarbose and vigibose are accepted as powerful inhibitors on α-amylase and α-glucosidase for DM. On the other hand, several reports have revealed that the synthetic enzyme inhibitors have certain adverse effects including liver damage, gastrointestinal disturbances [3–6]. From this point of view, many efforts are performed to search for more effective and safe inhibitors of key enzymes from plants to develop natural agents to treat these diseases.

Human environment consistently encounters with mutagenic and carcinogenic agents and eradication of these agents appear to be very laborious. Recently, it has been accepted that plants and their products demonstrate one of the main sources for compounds with antimutagenic potential and, indeed, several secondary plant metabolites have demonstrated chemo preventive activity against to genotoxic agents [7]. These antimutagens and anticarcinogens may inhibit one or more stages of the carcinogenic process and prevent or delay the formation of cancer. In addition to all these situations it should be noted that infectious diseases and the increasing antibiotic resistance are major global problems which threaten human health. Due to the indiscriminate usage of commercial antimicrobial drugs, multi-drug resistance in both human and plant pathogenic microorganisms has developed. Therefore scientists have tried to notice new antimicrobial and antimutagenic substances from various sources including plants [8]. It is known that, now natural products and their derivatives hold more than 50% of all the drugs in clinical usage with one quarter originating from higher plants.

The genus Cotoneaster (Rosaceae) comprises 85 species, which are predominately distributed around the Europe and Asia [9]. The genus is represented in Turkey by 8 species [10]. Several species of Cotoneaster are used to medicinal purposes such as cardioactive, diuretic, expectorant and antiviral in different countries [11,12]. Cotoneaster nummularia, called “Dağ müşmü-
las or ‘Tavşan elması’, is perennial herbs and grows in Anatolia. The medicinal uses of the species range from cures for diabetes mellitus and hemorrhoids, to being used as an expectorant in Anatolia folk medicine [13–15]. Although this plant has numerous applications on herbal remedy, thus far there are no scientific evidences behind the uses. In view of the above, the goal of this study was to thoroughly examine the composition (especially phenolic components) and biological activities (anti-oxidant, anti-bacterial, mutagenic/anti-mutagenic, anti-cholinesterase, anti-tyrosinase and anti-diabetic (α-amylase and α-glucosidase) of different solvent extracts from C. nummularia which is a wild plant.

Materials and Methods

Plant Material

*Cotoneaster nummularia* was collected from Konya-Turkey (Yukseken village, dry slopes) Taxonomic identification of the plant material was confirmed by the senior taxonomist Dr. Murad Aydin Sanda, from the Department of Biology, Selcuk University. The voucher specimen has been deposited at the Herbarium of the Department of Biology, Selcuk University, Konya-Turkey.

Ethics Statement

For the collection of plants, no specific permits were required for the described field studies. For any locations/activities, no specific permissions were required. All locations where the plants were collected were not privately-owned or protected in any way and the field studies did not involve endangered or protected species. This study was approved by the University of Selcuk institutional review board.

Extraction

To produce solvent extracts, the air-dried samples (10 g) of the twigs of *Cotoneaster nummularia* were extracted with 250 mL of solvents (ethyl acetate or methanol) in a Soxlet apparatus for 6–8 h. The extracts concentrated under vacuum at 40°C by using a rotary evaporator. For water extract, the air-dried samples (5 g) were boiled with 250 mL of distilled water for 30 min. The water extract was filtered and lyophilized (~80°C, 48 h). Extracts were stored at +4°C in dark until use. Extracts obtained using organic solvents were dissolved in methanol and then filtered. Water extract was dissolved in water at different concentrations.

Quantification of Phenolic Compounds by RP-HPLC

Phenolic compounds were evaluated by reversed-phase high-performance liquid chromatography (RP-HPLC). Detection and quantification were carried out with a LC-10ADpump, a Diode Array Detector, a CTO-10Avp column heater, DGU-14A degasser and SIL-10ADvp auto sampler. Separations were conducted at 30°C on C-18 reversed-phase column (250 mm × 4.6 mm length, 5 μm particle size). The eluates were diluted at 278 nm. The mobile phases were A: 0.02% phosphoric acid in distilled water and B: methanol. For analysis, the samples were dissolved in methanol, and 20 μL of this solution was injected into the column. The elution gradient applied at a flow rate of 0.8 mL/min was: 95% A/7% B for 0.1 min, 72% A/28% B in 20 min, 75% A/25% B in 8 min, 70% A/30% B in 7 min and same gradient for 15 min, 67% A/33% B in 10 min, 58% A/42% B in 2 min, 50% A/50% B in 8 min, 30% A/70% B in 3 min, 20% A/ 80% B in 2 min 100% B in 5 min until the end of the run. Phenolic compositions of the extracts were determined by a modified method of Caponio et al. [16]. Gallic acid, protocatechuic acid, (+)-catechin, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, (+)-epicatechin, syringic acid, vanillin, p-coumaric acid, ferulic acid, sinapinic acid, benzoic acid, α-coumaric acid, rutin, naringin, hesperidin, rosmarinic acid, eriodictyol, trans-cinnamic acid, quercetin, naringenin, luteolin, kaempferol and apigenin were used as standard. Identification and quantitative analysis were done by comparison with standards. The amount of each phenolic compound was expressed as mg per gram of the extract.

Determination of Total Bioactive Components

**Total phenolic content.** The total phenolic content was determined by employing the methods given in the literature [17] with slight modification. Sample solution (0.25 mL) was mixed with diluted Folin-Ciocalteu reagent (1 mL, 1:9) and shaken vigorously. After 3 min, Na2CO3 solution (0.75 mL, 1%) was added and the sample absorbance was read at 760 nm after 2 h incubation at room temperature. The total phenolic content was expressed as equivalents of gallic acid (mgGAEs/g extract) according to the equation obtained from the standard gallic acid graph.

**Total flavonoid content.** The total flavonoid content was determined using the Dowel method as adapted by Berk et al. [18]. Briefly, sample solution (1 mL) was mixed with the same volume of aluminium trichloride (2%) in methanol. Similarly, a blank was prepared by adding sample solution (1 mL) to methanol (1 mL) without AlCl3. The sample and blank absorbances were read at 415 nm after 10 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The total flavonoid content was expressed as equivalents of rutin (mgREs/g extract) according to the equation obtained from the standard rutin graph.

**Total Antioxidant Activity**

**Phosphomolybdenum method.** The total antioxidant activities of the samples were evaluated by phosphomolybdenum method according to Berk et al. [18] with slight modification. Sample solution (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample absorbance was read at 695 nm after a 90 min incubation at 95°C. The total antioxidant capacity was expressed as equivalents of ascorbic acid (mgAEs/g extract) as determined by the equation obtained from the standard ascorbic acid graph.

**β-carotene–linoleic acid method.** In this assay antioxidant activity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [19] with slight modification. A stock solution of β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in chloroform (1 mL, HPLC grade). 25 μL linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL of oxygenated distilled water was added with vigorous shaking; 1.5 mL of this reaction mixture was dispersed to test tubes and sample solution (0.50 mL, 2 mg/mL) were added and the emulsion system was incubated for up to 2 h at 30°C. The same procedure was repeated with the standard (BHA) and a blank. After this incubation period, the sample absorbance was read at 490 nm. Measurement of absorbance was continued until the color of β-carotene disappeared. The bleaching rate (R) of β-carotene was calculated according to Eq. (1).
Radical Scavenging Activity

Free radical scavenging activity (DPPH). The effects of the samples on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were estimated according to Sarikurkcu [20]. Sample solution (1 mL) was added to 4 mL of a 0.004% methanol solution of DPPH. The sample absorbance was read at 517 nm after a 30 min incubation at room temperature in dark. Inhibition of free radical DPPH in percent (I%) was calculated in following way: (Eq. (3))

\[
I(\%) = \left(1 - \frac{A_{sample}}{A_{control}} \right) \times 100
\]

where \(A_{control}\) is the absorbance of the control reaction (containing all reagents except the test compound) and \(A_{sample}\) is the absorbance of the test compound. BHA was used as a control. 50% of free radical inhibition (IC50) of samples was calculated. The lower the IC50 value indicates high antioxidant capacity.

ABTS (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging activity. The scavenging activity against ABTS cation radical was measured according to the method of Re et al. [21] with slight modification. Briefly, ABTS+ radical cation was produced directly by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to stand for 12–16 h at dark at the room temperature. Prior to beginning the assay, ABTS solution was diluted with methanol to an absorbance of 0.700 (a=30, 60, 90, 120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Eq. (2).

\[
AA = \frac{(R_{control} - R_{sample})}{R_{control}} \times 100
\]

Reducing Power

Cupric ion reducing method (CUPRAC assay). The cupric ion reducing activity (CUPRAC) was determined according to the method of Apak et al. [23]. Sample solution (0.5 mL) was added to premixed reaction mixture containing CuCl₂ (1 mL, 1 M, pH 7.0), neocuproine (1 mL, 7.5 mM) and NH₄Ac buffer (1 mL, 1 M, pH 7.0). Similarly, a blank was prepared by adding sample solution (0.5 mL) to premixed reaction mixture (3 mL) without CuCl₂. Then, the sample and blank absorbances were read at 450 nm after a 30 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The EC50 value (the effective concentration at which the absorbance was 0.5) was calculated for extracts and BHA.

Metal Chelating Activity on Ferrous Ions

The metal chelating activity on ferrous ions was determined by the method described by Akmusek et al. [25]. Briefly, sample solution (2 mL) was added to FeCl₂ solution (0.05 mL, 2 mM). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL). Similarly, a blank was prepared by adding sample solution (2 mL) to FeCl₂ solution (0.05 mL, 2 mM) and water (0.2 mL) without ferrozine. Then, the sample and blank absorbances were read at 562 nm after 10 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. The metal chelating activity was expressed as equivalents of EDTA (mgEDTA/g extract) according to the equation obtained from the standard EDTA graph.

Enzyme Inhibitory Activity

Cholinesterase inhibition. Cholinesterase (ChE) inhibitory activity was measured using Ellman’s method, as previously reported [25] with slight modification. Sample solution (50 µL) was mixed with DTNB (5,5-dithio-bis(2-nitrobenzoic) acid) (125 µL) and AChE (acetylcholinesterase) or BChE (butyrylcholinesterase) solution (25 µL) in Tris-HCl buffer (pH 8.0) in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of acetyl thiocione iodide (ATCI) or butyryl thiocione chloride (BTCI) (25 µL). Similarly, a blank was prepared by adding sample solution to all reaction reagents without enzyme (AChE or BChE) solution. The sample and blank absorbances were read at 405 nm after 10 min incubation at 25°C. The absorbance of the blank was subtracted from that of the sample and the cholinesterase inhibitory activity was expressed as equivalents of galanthamine (mgGALAES/g extract).

Tyrosinase inhibition. Tyrosinase inhibitory activity was measured using the modified dopachrome method with L-DOPA as substrate, as previously reported [26] with slight modification. Sample solution (25 µL) was mixed with tyrosinase (40 µL) and phosphate buffer (100 µL, pH 6.8) in a 96-well microplate and incubated for 15 min at 25°C. The absorption of the blank was subtracted from that of the sample and the tyrosinase inhibitory activity was expressed as equivalents of kojic acid (mgKAEs/g extract).

α-amylase inhibition. α-amylase inhibitory activity was performed using Caraway-Somogyi iodine/potassium iodide
from an overnight culture (1×10^9 cells/mL) was extracted by the method of Dean et al. The extract was used in the experiments. The aminoanthracene (2-AA, 20 μg/plate) was added to a suspension including 1×10^9 bacteria, 100 μL of top agar containing 10% of histidine/biotin (0.5 mM) for both Salmonella strains, mixed by vortex for eight seconds and poured onto the surface of minimal glucose agar plate. In the antimutagenicity test, the inhibitions of mutagenic activities of 4-NNPDA without S9 metabolic activation and 2-AF with S9 for S. typhimurium TA 98, SA in the absence of S9 mix and 2-AA in the presence of S9 mix for S. typhimurium TA 100 by the plant extract samples were determined. His-revertants were counted after incubation of the plates at 37°C for 48–72 h. Each sample was assayed using triplicate plates and the data presented as mean ± SD of two independent assays. The number of revertant colonies grown on plates containing the mutagen without plant extract was defined as 100% with 0% inhibition. The percentage of inhibition was calculated according to the formula: ([A-B]/[A-C])× 100, where A = No. of his. revertants in the absence of sample, B = No. of his. revertants in the presence of sample, C = spontaneous revertants [31]. Inhibition rate of 40% or more was defined as strong antimutagenicity, 25–40% inhibition as moderate antimutagenicity. Inhibitory effects of less than 25% were considered as weak and were not recognized as a positive result [32].

### Antimutagenicity Assay

Strains of Escherichia coli ATCC 25922, Bacillus cereus ATCC 11778, Pseudomonas aeruginosa ATCC 27853, methicillin sensitive Staphylococcus aureus ATCC 25923 (MSSA), Klebsiella pneumoniae ATCC 70063, Salmonella enteritidis ATCC 13076, Streptococcus pneumoniae ATCC 10015, Sarcina lutea ATCC 9341, Enterococcus faecalis ATCC 29212, methicillin resistant Staphylococcus aureus ATCC 43300 (MRSA) for the determination of antibacterial activities, and strains of methicillin resistant Staphylococcus aureus isolated from the clinical samples, for the detection of anti-MRSA activities of Cotoneaster nummularia extracts were used. The methicillin resistances of strains were determined by agar screening, oxacillin disc diffusion and broth microdilution methods. The standard microorganisms were gotten access to from Microbiology Research Laboratory, Department of Biology, Selcuk University, and Konya, Turkey. Standard and isolated strains of bacteria were grown to exponential phase in Brain-Heart Infusion Broth at 37°C overnight with aeration. The cultures were then plated on Mueller-Hinton Agar, overnight at 37°C with aeration. Then, approximately five colonies of cultures suspended in sterile physiologic water. The bacterial suspensions were adjusted to 0.5 McFarland standard turbidity (10^5 CFU/mL). Finally, these suspensions used as inoculums were prepared at 10^5 CFU/mL by diluting fresh cultures at McFarland 0.5 density. The broth microdilution method was employed for antibacterial and anti-MRSA activity tests. Mueller-Hinton Broth (100 μL) was placed into each 96 wells of microplates. Extract solutions initially prepared at a concentration of 10 mg/ml were added into first wells of microplates and two fold dilutions of the extracts (2.5–0.00122 mg/ml) were made by dispensing the solutions to the remaining wells. Then, 100 μL of culture suspensions were inoculated to each well. Gentamicin and Oxacillin were used as positive control. The sealed microplates were incubated at 35°C for 18 h. Microbial growth was determined by adding 20 μL of 2,3,5-Triphenyl-tetrazolium chloride (0.5%) after incubation to each well and incubating for 30 minute at 37°C [33]. The lowest concentration of the extracts that completely inhibit macroscopic growth was determined as minimum inhibitory concentrations (MICs).
Antioxidant Potentials and Phytochemical Composition

Antioxidant capacities of ethyl acetate, methanol and water extracts from *C. nummularia* were evaluated by free radical scavenging assay (DPPH, ABTS and O₂⁻), reducing power, phosphomolybdenum, β-carotene/linoleic acid bleaching and metal chelating assays. Free radical scavenging activities expressed as IC₅₀ values and the results are summarized in Table 1. BHA was the reagent used as standard in these assays. Generally, methanol and water extracts showed the highest activity with lower values of IC₅₀. Occurrence of the highest DPPH and O₂⁻ scavenging activities were observed in the water extract of the plant, followed by the methanol extract. However, the greatest ABTS inhibition was caused by the methanol extract (IC₅₀=0.020 mg/ml) closely followed by the water extract (IC₅₀=0.023 mg/ml). There were no significant differences in free radical scavenging activities between methanol and water extracts (p>0.05). As evidences by this table, ethyl acetate extract showed lowest activity with IC₅₀ values two times higher than methanol and water extracts. However, BHA has a stronger effect of scavenging free radicals than tested extracts in the test systems.

Reducing power activities were evaluated by using EC₅₀ (the effective concentration at which the absorbance was 0.5). Reducing powers of tested extracts appeared to be in the same manner with free radical scavenging activity (Table 1). In the ferric and cupric reducing power assays, the methanol and water extracts were the most effective even the methanol extract (EC₅₀=0.006 mg/ml) closely followed by the water extract (IC₅₀=0.023 mg/ml). There were no significant differences in free radical scavenging activities between methanol and water extracts (p>0.05). As evidences by this table, ethyl acetate extract showed reducing power activities by several investigators who indicated that methanol and water extracts were not significantly different (p>0.05), but these extracts showed significant difference in cupric reducing activity (p<0.05). These results are in accord with those reported by several investigators who indicated that methanol and water extracts the most active in free radical and reducing power assays [34,35]. Ethyl acetate extract has the lowest activity in both ferric and cupric reducing power assays (p<0.05).

Total antioxidant activities of tested extracts were investigated by phosphomolybdenum and β-carotene/linoleic acid assays. Phosphomolybdenum method is based on the reduction of Mo (IV) to Mo (V) by the antioxidants and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. In this assay, the highest activity was demonstrated by water extract (177.2 mgAEs/g) followed by methanol (161.26 mgAEs/g) and ethyl acetate extract (56.06 mgAEs/g). Apparently, the total antioxidant activity of water extract was 3.16 fold higher than that of ethyl acetate (Table 1). However, differences in the ascorbic acid equivalent values of methanol and water extracts were not significant (p>0.05). Our results are concomitant with previous findings where superior total antioxidant activities in water extracts of several plants compared to other extracts was reported [36,37]. The potential of the extracts to inhibit lipid peroxidation was evaluated using the β-carotene/linoleic acid bleaching assay. In terms of inhibition ability of linoleic acid oxidation, those samples can be ranked from high to low in the following order: BHA (95.07%), methanol (85.46%), and water extract (71.79%).

Results and Discussion

Statistical Analysis

For all the experiments all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s honestly significant difference post hoc test with α = 0.05. This treatment was carried out using SPSS v. 14.0 program.

| Table 1. Antioxidant activities of three extracts from *Cotoneaster nummularia* (mean± SD). |
| --- |
| **Antioxidant Capacity** | **Metal Chelating Activity** | **Ferrous ion Chelating Activity** |
| **Solvent** | **DPPH** | **ABTS** | **O₂⁻** | **IC₅₀ (mg/μL)** | **EC₅₀ (mg/μL)** | **CUPRAC** | **β-carotene/linoleic acid assay (mgEDTAEs/g)** | **Phosphomolybdenum** |
| Ethyl acetate | 0.03±0.01 | 0.04±0.01 | 0.02±0.01 | 1.09±0.04 | 1.03±0.04 | 1.64±0.06 | 177.2±3.6 | 56.06±2.7 |
| Methanol | 0.04±0.01 | 0.03±0.01 | 0.02±0.01 | 1.04±0.04 | 1.05±0.04 | 1.05±0.06 | 161.26±4.8 | 56.06±3.1 |
| Water | 0.05±0.01 | 0.02±0.01 | 0.01±0.01 | 1.06±0.04 | 1.02±0.04 | 1.06±0.06 | 177.29±1.7 | 56.06±3.6 |
| BHA | 0.01±0.01 | 0.01±0.01 | 0.01±0.01 | 0.02±0.01 | 0.02±0.01 | 0.02±0.01 | 95.07±0.5 | 56.06±2.7 |

*In same column marked with different letters indicate significant difference (p<0.05).*

**AEs**: ascorbic acid equivalents.

**EDTAEs**: EDTA equivalents.

**nt**: no tested.
Water (93.04%) > Methanol (89.63%) > Ethyl acetate (85.46%). Nonetheless, inhibition values of methanol and water extracts were not statistically different ($p > 0.05$). Water extract exhibited marked antioxidant ability close to that of BHA. Hence, the water extract could be considered as a natural inhibitor for prevention of lipid oxidation in food industry. It is probable that the antioxidant components such as phenolics in the water extract can reduce the extent of $\beta$-carotene destruction by inhibiting the linoleic acid oxidation in the test system.

Metal ion chelating activity of each extract was tested against ferrous ion. The chelating activities of the extracts were evaluated using EDTA as a standard (mg EDTA/g extract). In accordance with results of other antioxidant assays, potent chelation abilities were again detected in the water extracts with 18.66 mg EDTA/s/g (Table 1) ($p < 0.05$). Since ferrous ions were the most effective pro-oxidant for lipid peroxidation, the water extract from <i>C. nummularia</i> would be beneficial. In fact, numerous studies indicated that some plant extracts are capable of with and stabilizing ferrous ion and rendering it’s unable to participate in metal-catalyzed initiation and hydroperoxide decomposition reactions [27,38-39].

Total phenolic contents of the <i>Cotoneaster</i> extracts ranged from 81.11 to 266.39 mgGAES/g extracts. Water extract had the highest total phenolics, followed by methanol and ethyl acetate. Similarly, total flavonoid contents were found to be significantly high in water and methanol extracts. Differences in the total phenolic and flavonoid content of methanol and water extracts were not significant ($p > 0.05$). Based on these results, the flavonoid content of methanol extract was three times higher than that on the ethyl acetate extract (Figure 1) ($p < 0.05$). The results of total phenolic content showed a similar tendency to those of the antioxidant abilities. In this direction, the high content of total phenolics in methanol and water extracts might be explain the strong antioxidant abilities of the extracts. These results are in accordance with other reports in the literature, which showed strong relationship between antioxidant activities and total phenolic content [40-42].

Although a number of analytical methods have been proposed for the separation and determination of phenolic compounds, high performance liquid chromatography (HPLC) technique with diode array detector has become a dominant procedure since it has some advantages such as simple sample treatment, short analysis time and high reproducibility [43]. Using HPLC analysis, the content of the principal phenolic compounds of plant extracts are listed in Table 2. 25 standard phenolics were analyzed and 16 of them were identified in the extracts (Figure 2). Gallic acid, quercetin, naringenin, hesperidin, sinapic acid, $\alpha$-coumaric acid, rutin, naringin and kaempferol were not detected in these extracts (data is not shown in Table 2). Major phenolic compounds were determined as ferulic acid (22.60–28.36 mg/g extract), chlorogenic acid (5.70–16.66 mg/g extract), (-) epicatechin (5.24–6.92 mg/g extract) and catechin (3.26–6.14 mg/g extract). These data established that the biological activities of <i>C. nummularia</i> could be attributed to their polyphenol compounds. Recent studies showed that ferulic acid acts as a potent antioxidant by scavenging free radicals. In addition, ferulic acid proposed as a potential treatment for many disorders including Alzheimer’s diseases, diabetes mellitus and skin diseases [44-46]. Again, it possesses a wide spectrum of antimicrobial (Gram-positive bacteria, Gram-negative bacteria and yeasts) and antimutagenic activity [47,48]. Thus, high concentration of ferulic acid was thought to be responsible for the biological activities exerted. Actually, the higher ferulic acid content in methanol extract conferred to its strong biological activities. Moreover, chlorogenic acids, (+)-catechin and (-)-epicatechin content were closely correlated to biological activities such as metal chelating and inhibition of lipid peroxidation [49]. In this way, the superiority of methanol and water extracts biological activities may be explained by the amount and nature of these major phenolic compounds.

**Enzyme Inhibitory Activities**

The ethyl acetate, methanol and water extracts were tested for AChE, BChE, tyrosinase, $\alpha$-amylase and $\alpha$-glucosidase inhibitory activities using colorimetric methods in a 96-well microplate. These activities were expressed as equivalents of standard inhibitors (galatamine for AChE and BChE, kojic acid for tyrosinase and acarbose for $\alpha$-amylase and $\alpha$-glucosidase). The results are shown in Table 3. The methanol and water extracts exhibited remarkable AChE inhibitory activity with 4.30 and 4.77 mgGALAEs/g extract, respectively. However, water extract had the lowest inhibitory activity on BChE. With regard to tyrosinase inhibitory activity, water extract showed a good inhibitory activity with 32.31 mg KAEs/g. The tyrosinase inhibitory activity of ethyl acetate (24.22 mg KAEs/g) is slightly different from that of the methanol extract (24.01 mg KAEs/g). Methanol extract possessed very good inhibitory activity against $\alpha$-glucosidase and $\alpha$-amylase. In $\alpha$-glucosidase inhibition assay the activity was methanol > water > ethyl acetate. In contrast to $\alpha$-glucosidase assay, the lowest $\alpha$-amylase inhibition activity was revealed by water extract. To summarize, methanol and water extracts had the higher inhibitory activity on tested enzymes compared to ethyl acetate extract. The effective inhibitory

![Figure 1. Total phenolic and flavonoid content in three extracts from Cotoneaster nummularia (mean±SD).](image-url)

*Note: In each bar different letters indicate significant difference ($p < 0.05$). (GAEs: Gallic acid equivalents; REs: Rutin equivalents). doi:10.1371/journal.pone.0113527.g001*
activities of these samples were most likely due to their high phenolic content, especially ferulic acid. Similar results were reported for Impatiens bicolor and selected Lamiaceae species by Shahwar et al. [50] and Vladimir-Knezevic et al. [51], respectively. Many phenolic compounds (ferulic acid, catechin eg.) were reported to protect against the above mentioned public health problems [52,53]. On the other hand, a number of studies were figured that no correlation was between phenolic content/phenolic components and enzyme inhibitory activities [54,55]. This situation can be explained the complex nature of phytochemicals and their synergistic or antagonistic effects. The present study reports, for the first time, on the in vitro enzyme inhibitory activities of the different extracts of Cotoneaster nummularia.

Anti-bacterial Activity
In this study antibacterial and anti-MRSA activities of water, methanol and ethyl acetate extracts of Cotoneaster were investigated by broth microdilution method according to Koç et al. [33]. The obtained results are presented in Table 4.

Water extract was found to be remarkable antibacterial against gram positive microorganisms. The MIC values were determined as 0.625 mg/ml for S. aureus (MSSA), S. aureus (MRSA), and S. lutea. MSSA, MRSA and S. lutea were affected by control antibiotic at concentrations of 0.00244, 0.07812 and 0.00244 mg/ml, respectively. It has been seen that water extract revealed a significant effect against MRSA. While E. faecalis was the most sensitive bacterium, B. cereus and S. pneumoniae were resistant Gram-positive bacteria against water extract. The MIC value of water extract was determined as 0.039 mg/ml against E. faecalis. Although E. coli was affected by water extract at a 0.625 mg/ml dose, K. pneumoniae, S. enteritidis, and P. aeruginosa were found to be resistant to this extract. As can be clearly seen from the Table 4 Gram-negative microorganisms were more resistant than Gram-positive bacteria against water extract of Cotoneaster.

Methanol extract exhibited significant antibacterial activity against E. faecalis at a concentration of 0.312 mg/ml. The MIC values of methanol extracts were determined as 2.5 mg/ml against E. coli, P. aeruginosa, MSSA, and MRSA. B. cereus, K. pneumoniae, S. lutea, and S. enteritidis were not affected by this extract at all test doses. The MIC value was determined as 0.625 mg/ml for S. pneumoniae. While P. aeruginosa and S. pneumoniae were found to be resistant to water extract, they were affected by methanol extract. However, MIC values of the water extract were lower than those of methanol extract. Except for MRSA strain 8, the ethyl acetate extract of Cotoneaster exhibited antimicrobial activity at a concentration of 2.5 mg/ml against both standard and isolated bacteria tested in the present study. The MIC value was determined as 1.25 mg/ml for MRSA strain 8. It was concluded that E. faecalis was the most sensitive bacteria and B. cereus, K. pneumoniae, and S. enteritidis were the most resistant bacteria against Cotoneaster extracts except for ethyl acetate extract.

Table 2. Phenolic components in the solvent extracts from Cotoneaster nummularia (mg/g extract) (mean±SD).

| No | Phenolic Components | Ethyl Acetate | Methanol | Water |
|----|---------------------|---------------|----------|-------|
| 1  | Protocatechuic acid | 1.64±0.04a    | Nd       | 1.16±0.04b |
| 2  | (+)- Catechin        | 6.14±0.16a    | 4.58±0.14b | 3.26±0.12c |
| 3  | p-Hydroxybenzoic acid| 1.08±0.04b    | 0.94±0.04b | 3.16±0.04a |
| 4  | Chlorogenic acid     | 5.70±0.12c    | 16.66±0.26a | 13.92±0.24b |
| 5  | Caffeic acid         | 0.54±0.01a    | 0.56±0.01a | Nd |
| 6  | (+)- Epicatechin     | 5.24±0.32a    | 6.50±0.32a | 6.92±0.32a |
| 7  | Syringic acid        | 0.12±0.01     | Nd       | Nd |
| 8  | Vanillin             | 0.08±0.01     | Nd       | Nd |
| 9  | p- Coumaric acid     | 0.26±0.02b    | 0.48±0.01a | 0.18±0.01c |
| 10 | Ferulic acid         | 25.54±0.62ab  | 28.36±0.62a | 22.50±0.62b |
| 11 | Benzoic acid         | 1.32±0.04a    | Nd       | 1.28±0.04a |
| 12 | Rosmarinic acid      | 0.14±0.01b    | Nd       | 0.28±0.01a |
| 13 | Eriodictyol          | 1.16±0.01a    | 0.28±0.01c | 1.00±0.01b |
| 14 | trans-Cinnamic acid  | 0.16±0.02b    | 0.24±0.02a | 0.02±0.01c |
| 15 | Luteolin             | 0.08±0.01     | Nd       | Nd |
| 16 | Apigenin             | 0.22±0.01a    | Nd       | 0.04±0.01b |

In same row marked different letters indicate significant difference (p<0.05); nd, not determined.
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Biological Activities of Cotoneaster nummularia

(a)

(b)

(c)
extract. Our results revealed that ethyl acetate extract of plant had antibacterial and anti MRSA activities at a dose of 2.5 mg/ml except for MRSA strain 8.

Mutagenic/Antimutagenic Activity

In this study, the mutagenic and antimutagenic activities of Cotoneaster water extract (with doses of 10000 μg/plate and lower) were investigated. The assays were performed using the standard plaque incorporation method defined by Maron and Ames [29]. The revertant colony numbers observed in the mutagenicity assay were determined and are given in Table 5.

As seen in Table 5, spontaneous revertants were within normal values in all strains examined. The average revertant colony numbers in negative control were 40 ± 8 for TA98 and 115 ± 6 for TA100 with S9 and 22 ± 1 and 118 ± 6 without S9, respectively (p > 0.05). When application of S9 in TA98 was increased revertant colony numbers, application of S9 in TA100 was decreased revertant colony numbers (p > 0.05). On the contrary, the plates with the positive control mutagens (SA, 2-AF, 2-AA and 4-NPDA) showed significant increases relative to the spontaneous mutation rate in the two tested strains. Most of the results, increasing or decreasing relative to negative control group, were not statistically significant at p < 0.05 (Tamhane) in examined strains. In order to establish a dose-response relationship, 5 different concentrations of Cotoneaster extract were tested, and no induced revertants were observed along the dose range tested in either with or without S9 with two strains. The results of the present study showed that all test doses of Cotoneaster extract were not found to be mutagenic for S. typhimurium TA98 and TA100 in the presence and absence of S9 mix.

The revertant colony numbers observed in the antimutagenicity assay and inhibition (%) rates of the extracts were given in Table 6. Cotoneaster extract exhibited moderate antimutagenic activities at doses of 10000, 5000 and 1000 μg (32%, 33%, and 31%, respectively) against 4-NPDA in the absence of S9 mix in S. typhimurium TA98 (Table 6). It was determined that thrombocytopenia and 2-AF, 2-AA and 4-NPDA showed significant increases relative to the spontaneous mutation rate in the two tested strains. Most of the results, increasing or decreasing relative to negative control group, were not statistically significant at p < 0.05 (Tamhane) in examined strains. In order to establish a dose-response relationship, 5 different concentrations of Cotoneaster extract were tested, and no induced revertants were observed along the dose range tested in either with or without S9 mix in TA100 strain (Table 6). It was determined that there was no induced inhibition ratios were observed along the dose range tested in the absence of S9 mix. On the other hand, 100 and 10 μg doses of the extract were found to be weak antimutagenic with a ratio of 19% and 20%, respectively. While Cotoneaster extracts showed strong antimutagenicity at doses of 10000 (50%) and 5000 (49%) against 2-AA; 1000, 10 and 10 μg doses of the extract exhibited moderate antimutagenic activities in the presence of S9 mix in TA98 strain with a ratio of 40%, 29%, and 25%, respectively (p < 0.05). It was determined that metabolic activation enzymes (S9 mix) induced the inhibition ratios of the extract compared to those of extracts in the absence of S9 and a dose-response relationship was observed along the dose range tested.

It was seen that Cotoneaster extract manifested moderate antimutagenicity at concentrations of 10000, 5000, 1000, and 100 μg (40%, 38%, 36%, and 27%, respectively) against SA, while 10 μg dose of extract was found to be weak antimutagenic with a ratio of 20% in the absence of S9 mix in TA100 strain (Table 6). Except for 100 and 10 μg, all doses tested exhibited strong antimutagenic activity against 2-AA in the presence of metabolic activation system (Table 6). The highest inhibition ratio (59%) was observed in 10000 μg/plate dose of the extract, followed by 100 μg/plate (58%) and 5000 μg/plate (55%). Meanwhile, the extracts at concentrations of 100 and 10 μg were found to be weak antimutagenic capacities with S9 in TA 100 strain.

Based on the results it was determined that Cotoneaster water extract had significant antimutagenic capacity in the presence of metabolic activation enzymes (S9) for TA98 at concentrations of 10000, 5000,1000, and 100 μg/plate against 2-AF, for TA100 strain at concentrations of 10000, 5000,1000 μg/plate against 2-AA.

Overall, it can be stated that the S9 enzymes increase the antimutagenic activities of Cotoneaster extracts significantly (Table 6). A possible cause of this can be explained by the following way: The antimutagenic response is activated by invoking the competitive inhibition by liver glycosides of P450 isoenzymes [56]. It has been previously determined that some plant metabolites are potent inhibitors of cytochrome c (P450) reductase [57]. Kappus [58] demonstrated that this preventive activity after metabolic activation is related to the function of cytochrome P450 isoforms in detoxification systems with reductase or oxygenase, whose function in the system is antioxidant scavenging, neutralizing compounds that generate oxygen radicals, free radicals and reactive oxygen species [59]. Thus, the greatest antimutagenic activity observed in assays in the presence of a metabolic fraction may be related to the activation of a cytochrome P450 which mediates the oxidation of promutagens [60], indicating that this action could be due to the competitive inhibition by glycosides of cytochrome P450, thus avoiding formation of the promutagen [56;61]. This plant may be natural source of antimutagenic agents and may be used in the pharmacology industry.

Table 3. Enzyme inhibitory activities of three extracts from Cotoneaster nummularia (mean ± SD).

| Solvents      | AChE (mgGALAEs/g)* | BChE (mgGALAEs/g)* | α-amylase (mgACAEs/g)** | α- Glucosidase (mgACAEs/g)** | Tyrosinase (mgKAEs/g)*** |
|---------------|--------------------|--------------------|-------------------------|-----------------------------|--------------------------|
| Ethyl Acetate | 4.07 ± 0.40a       | 5.46 ± 0.32a       | 7.91 ± 0.90b            | 51.02 ± 4.09b               | 24.22 ± 1.45a            |
| Methanol      | 4.30 ± 0.45a       | 6.03 ± 0.11a       | 13.62 ± 1.90a           | 82.34 ± 0.90a               | 24.01 ± 1.53a            |
| Water         | 4.77 ± 0.26a       | 0.65 ± 0.01b       | 1.90 ± 0.88c            | 69.54 ± 4.21c               | 32.31 ± 0.26b            |

*In same column marked with different letters indicate significant difference (p < 0.05).

*GALAEs: galanthamine equivalents.

**ACAEs: acarbose equivalents.

***KAEs: kojic acid equivalents.

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Table 5. Mutagenicity of Cotoneaster extract towards S. typhimurium TA98 and TA100 strains with or without S9.

| Concentration (µg/plate) | Number of His⁺ Revertants/plate |
|------------------------|---------------------------------|
|                        | TA 98                           | TA 100                          |
|                        | S9 (−)                          | S9 (+)                          | S9 (−)                          | S9 (+)                          |
| Negative Control       |                                 |                                 |                                 |                                 |
| 100 µ/plate            | 22±1 a                          | 40±8 a                          | 118±6 a                         | 115±6 a                         |
| Positive Control       |                                 |                                 |                                 |                                 |
| 744±98 b               | 4803±109 b                      | 1855±177 b                      | 3878±159 b                      |
| COTONEASTER WATER EXTRACT |                                |                                 |                                 |                                 |
| 0                      | 26±4 a                          | 40±5 a                          | 122±9 a                         | 115±6 a                         |
| 10000                  | 26±3 a                          | 45±7 a                          | 123±8 a                         | 132±9 a                         |
| 5000                   | 25±1 a                          | 47±2 a                          | 124±5 a                         | 117±18 a                        |
| 1000                   | 22±2 a                          | 53±7 a                          | 125±7 a                         | 123±5 a                         |
| 100                    | 25±6 a                          | 55±7 a                          | 120±10 a                        | 119±1 a                         |
| 10                     | 24±4 a                          | 55±3 a                          | 110±23 a                        | 109±16 a                        |

* Differences between groups having the same letter in the same column are not statistically significant (ANOVA, Tamhane, p>0.05).
* Negative control: Sterile distilled water (100 µl/plate) was used as negative control for S. typhimurium TA98 and TA100 both in the presence and absence of S9.
* Positive controls:
  2-Aminofluorene (5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; 4-nitro-O-phenylenediamine (20 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA98 strain.
  2-Aminanthracene (20 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; Sodium azide (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for S. typhimurium TA100.

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### Table 6. Antimutagenicity and inhibition rates of Cotoneaster extracts towards *S. typhimurium* TA98 and TA100 strains with and without metabolic activation (S9).

| Concentration (µg/plate) | Number of His\(^+\) Revertants/plate |  |  |  |  |  |
|--------------------------|---------------------------------------|---|---|---|---|---|
|                           | TA 98                                 | TA 100                          |
|                           | S9 (−) | % inhibition | S9 (+) | % inhibition | S9 (−) | % inhibition | S9 (+) | % inhibition |
| **Negative Control**      | 100 µl/plate | 38 | 50 | 114 | 50 | 114 | 50 |
| **Positive Control**      | 849 | 40 | 146 | 50 | 2546 | 50 | 3878 | 50 |
| **COTONEASTER WATER EXTRACT** | 0 | 48 | 50 | 140 | 50 | 114 | 50 |
| 10000                    | 607 | 32 | 2425 | 50 | 1586 | 40 | 1670 | 50 |
| 5000                     | 603 | 33 | 2471 | 49 | 1641 | 38 | 1795 | 55 |
| 1000                     | 619 | 31 | 2906 | 40 | 1685 | 36 | 1670 | 58 |
| 100                      | 717 | 19 | 3471 | 29 | 1900 | 27 | 3493 | 10 |
| 10                       | 710 | 20 | 3633 | 25 | 2060 | 20 | 3214 | 18 |

*Negative control: Sterile distilled water (100 µl/plate) was used as negative control for *S. typhimurium* TA98 and TA100 both in the presence and absence of S9.

**Positive controls:**
- 2-Aminofluorene (5 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; 4-nitro-O-fenilendiamine (20 µg/plate) was used as positive direct mutagen in the absence of S9 mix for *S. typhimurium* TA98 strain.
- 2-Aminoanthracene (20 µg/plate) was used as positive indirect mutagen in the presence of S9 mix; Sodium azide (5 µg/plate) was used as positive direct mutagen in the absence of S9 mix for *S. typhimurium* TA100.

\(\text{abcde}\) Differences between groups having the same letter in the same column are not statistically significant (ANOVA, Tukey HSD, \(p>0.05\)).

\(\text{fgh}\) Differences between groups having the same letter in the same column are not statistically significant (ANOVA, Tamhane, \(p>0.05\)).
Conclusions
To sum up, such detailed studies on chemical composition and biological activity of different extracts from *C. nummularia* have been conducted for the first time. Thus their chemical composition was determined indicating significant amounts of polyphenols, furfural acid in particular. Based on biological activity assays, antioxidant, anti-bacterial, mutagenic/anti-mutagenic, anti-cholesterolesterase, anti-tyrosinase, anti-amylose and anti-glucosidase of the extracts were revealed. Water and methanol extracts possess notable biological properties. A high positive correlation observed between phytochemical composition and biological activities. Moreover, the water extract showed no mutagenic effect when tested with Ames test. Our data showed that there was no uniform response within or between the bacterial strains in terms of susceptibility to antimicrobial compounds in the water, methanol, and ethyl acetate extracts of *Cotoneaster*. These kinds of differences in susceptibility among the microorganisms against antimicrobial substances in plant extracts may be explained by the differences in cell wall composition and/or inheritance genes on plasmids that can be easily transferred among bacterial strains. Results of the present study suggested that the extracts from *C. nummularia* can be exploited as a potential source of natural agents (antioxidant, antibacterial, antimutagenic, and enzyme inhibitors) for the management of oxidative stress and global health problems. The data provides strong scientific evidence for traditional uses of *C. nummularia*. Finally, new investigations are needed to test the biological effects of *C. nummularia* on human health.

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Author Contributions
Conceived and designed the experiments: AU GZ. Performed the experiments: AU GZ EG. Analyzed the data: AU GZ EG. Contributed reagents/materials/analysis tools: AU GZ AA. Wrote the paper: AU GZ EG.

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