RESEARCH ARTICLE

Cytotoxic Activity-Guided Isolation from *Achillea monocephala*, and Biological Activities of its Different Extracts

Turgut Taşkin¹*, Eray M. Güler², Şeyda Şentürk¹, Damla D. Çelik¹, Turan Arabacı¹ and Ümran S. Gürer³

¹Department of Pharmacognosy, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
²Department of Medical Biochemistry, School of Medicine, Bezmialem Vakıf University, Istanbul, Turkey
³Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Marmara University, Istanbul, Turkey

Abstract:

Background:
The genus *Achillea* is one of the most important genus of the Asteraceae family and many species of *Achillea* are used in traditional medicine to treat several ailments.

Aim:
The aim of the current research was to evaluate *in vitro* cytotoxic activities of n-hexan, chloroform, ethyl acetate and methanol extracts and to isolate the active compounds from the extract showing the strongest cytotoxic activity. In addition to this, it was aimed to evaluate the biological activities (cytotoxic, antioxidant, anti-urease, anticholinesterase, antimicrobial) of different extracts and active compounds from *Achillea monocephala*.

Methods and Materials:
The *in vitro* antioxidant, cytotoxic, anti-urease, anticholinesterase and antimicrobial activities of different extracts from *A. monocephala* aerial parts were examined. The structures of the active compounds were determined by NMR techniques, UV, IR and LC-MS/MS analysis and their biological potential was examined.

Results:
The chloroform extract showed strong and selective cytotoxic activity on the cancer cell lines (MDA-MB-231, MCF-7). Besides, this extract exhibited stronger antimicrobial activity than other extracts. Therefore, through activity-guided procedures, luteolin, naringenin and 8-hydroxy-salvigenin compounds were isolated from this extract. The methanol extract showed stronger antioxidant (DPPH, ABTS, CUPRAC) and anticholinesterase activity than other extracts. The n-hexan extract exhibited the highest anti-urease activity. In this study, it was determined that the isolated compounds had a strong biological activity. Naringenin compound had stronger ABTS radical cation scavenging and ferric reducing/antioxidant power, cytotoxic and antimicrobial activity than other compounds. 8-hydroxy-salvigenin compound showed the highest urease and acetylcholinesterase enzyme inhibition.

Conclusion:
The results of this study suggest that the extracts and isolated compounds from the *A. monocephala* may be used as antioxidant, cytotoxic, anti-urease, anticholinesterase and antimicrobial agents in the future.

Keywords: *A. monocephala*, Biological activity, Luteolin, Naringenin, 8-hydroxy-salvigenin, Cytotoxic.

Article History
Received: April 07, 2020  Revised: May 18, 2020  Accepted: May 18, 2020

1. INTRODUCTION

The genus *Achillea* is one of the most important genus of the Asteraceae family and has more than 100 species that grow wild in different regions of the world. The main habitats of this genus are concentrated in different parts of Iran, Turkey, Serbia and Eastern regions of Europe. The phytochemical studies of *Achillea* species reveal that this genus contains many components (flavones, guaianolides, lignans, non-saturated carboxylic acids, phenolic glycosides, phthalate derivatives, piperidine amides, polyacetylenes, proazulenones, sesquiterpene
lactone diol, sesquiterpene lactones, sesquiterpenes, spirodepressolide, tannins, triterpene alkamides). Since the genus Achillea is widespread all over the world, many species of this genus have been used as traditional herbal medicines by the local population. It is known that there are many reports highlighting the ethnopharmacological uses of different species of Achillea. Many species of Achillea have been used as tonic, anti-inflammatory, anti-spasmodic, diaphoretic, diuretic and emmenagogic agents, wound healing and carminative. Achillea species are among the most important indigenous economic plants of Anatolia. Herbal teas prepared from some species of this genus are traditionally used in the treatment of abdominal pain and flatulence in Turkey [1 - 3]. Globally cancer is one of the diseases that has seriously affected the human population. In recent years, natural compounds which are thought to have less toxic side effects than current treatments have attracted the attention of researchers. The number of studies on the use of compounds and extracts isolated from medicinal plants instead of synthetic compounds has increased in recent years [4 - 6].

2. MATERIALS AND METHODS

2.1. Chemical Reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin–Ciocalteu (FCR), ascorbic acid, acetylcholinesterase (AChE), galantamine were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade. The column chromatography (CC) was achieved by using silica gel 0.2-0.5-mm (Sigma-Aldrich, USA) as stationary phases. Kieselgel 60 F(254) was used for thin layer chromatography. Fetal bovine serum (FBS), Leibovitz’s L15 Medium, McCoy’s 5a Medium, RPMI 1640 Medium, Eagle’s Minimum Essential Medium (E’MEM), penicillin-streptomycin, cholera toxin, and transferrin were obtained from Sigma-Aldrich (Seelze, Germany). MEGM Kit was obtained from Lonza/Clonetics Corporation (Basel, Switzerland).

2.2. Spectroscopic Methods

The NMR spectroscopic analyses were performed on a Bruker Avance III (500 MHz). MS analyses were performed using Q-TOF-LC/MS (Agilent6530, CA, US). Shimadzu UV-1800 and FT-IR Affinity-1 spectrophotometer was used for UV and IR spectra, respectively.

2.3. Plant Material and Preparation of Plant Extracts

The aerial parts of Achillea monopapheala Boiss. et Bal. were authenticated by Prof. Dr. Turan Arabaci. A voucher specimen (TA3042) was deposited at the herbarium of the Faculty of Pharmacy, İnönü University, for future reference.

2.4. Extraction and Isolation

The plant aerial parts were dried in shade (25°C) and grounded in a mechanical grinder (Renas, RBT1250) to a fine powder. The powdered sample (350 g) was extracted with organic solvents such as n-hexane, chloroform, ethyl acetate and methanol using the maceration method, respectively, until colorless. The organic phase was evaporated to dryness under reduced pressure. All the extracts obtained were stored at 4 °C for future analysis. The chloroform subextract (8 g) was submitted to silica gel column and eluted with n-hexane, chloroform and methanol mixture of increasing polarity, n-hexan (100%, 700 mL), n-hexan: chloroform (30%, 50%, 2x500 mL), chloroform (100%, 700 mL), chloroform: methanol (30%, 50%, 2x500 mL), methanol (100%, 500 mL), to obtain thirteen fractions. After these sub-fractions were controlled by TLC, similar ones were combined (Fr.I-V). Fr. I (0.40 g) was applied to a silica gel column using mixtures of toluene: ethyl acetate (7:3) to yield luteolin (4.3 mg). Fr. III was applied to preparative TLC using mixtures of toluene: ethyl acetate: methanol (70:30:10) to yield naringenin (6.2 mg). Fr. V was applied to a silica gel column using mixtures of toluene: ethyl acetate (80:20; 70:30, 50:50) and ethyl acetate: methanol (80:20; 70:30, 50:50) to yield 8-hydroxy-salvigenin (5.1 mg).

2.5. Cytotoxic Activity of Different Extracts from Plant

The cell culture and maintenance: MDA-MB-231 cells (as standard cell line from human breast carcinoma cells), MCF-7 cells (as standard cell line from human breast carcinoma cells) and 184A1 (as standard human normal breast/epithelium cells) were obtained from American Type Cell Culture Collection (ATCC, Germany). MDA-MB-231 cells were cultured in Leibovitz’s L15 Medium, MCF-7 cells were cultured in E’MEM and 184A1 cells were cultured in MEBM. The medium was supplemented with 10% FBS, 100 U/mL of penicillin and 100 ng/mL of streptomycin. Trypan blue exclusion test was used for the estimation of the number of viable cells.

Cytotoxicity assay: The cytotoxic activity of extracts and isolated compounds on the cells were examined by ATP levels measured with luminescence test (Cell-Titer-Glo Luminescence Cell Viability Assay, Promega). Cells were seeded onto 96-well plates at a density of 1.5×10^4 cells per well and incubated overnight at 37°C in 5% CO₂. The medium was then changed with a fresh complete medium including varying concentrations of extracts and isolated compounds. Control cells were treated with 0.1% DMSO. Cells were left for incubation under humidified 5% CO₂ and 95% O₂ at 37°C for 24 hours. Afterward, the cells were rinsed with the culture medium and examined for ATP. Each sample was supplemented with 100 µL of the reagent (Cell Titer-Glo Luminescence Cell Viability Assay, Promega), mixed for 2 minutes and incubated for 10 minutes at room temperature. The results were evaluated with a luminometer (Varioscan Flash Multimode Reader, Thermo, Waltham, MA). The light emitted in the presence of ATP was measured as relative light units.
The intensity of the emitted light quantities is directly related to the ATP content in the sample being tested. Cell viability was expressed as a percentage relative to the negative control group indicated as 100%. Half-maximal growth inhibitory concentration (IC_{50}) values were calculated from non-linear regression analysis from concentration-response curves. Live/dead assay was performed to quantify live and dead cells as described previously. All experiments were repeated three times and the standard deviation was 5% [7].

2.6. Antioxidant Study

2.6.1. DPPH Radical Scavenging Assay

240 μL 0.1 mM DPPH solution was added to 10 μL sample of the extracts and active compounds. The prepared mixture was stirred for 1 min. and placed at 25°C for 30 min. The mixture absorbance was determined against the reference at 517 nm. The control sample was carried out under the same conditions using 10 μL of methanol instead of experimental and standard materials and the control sample was daily measured. The investigation was performed three times and the averages of the data and standard deviation were calculated. The data were given as IC_{50}/mg/mL [8].

2.6.2. ABTS Radical Cation Scavenging Assay

40 μL of extracts/active compounds were prepared and then 3960 μL of ABTS+ working solution was added on the prepared mixture. The mixture absorbance was determined against the reference at 734 nm for 6 min. The control sample was prepared under the same conditions with the use of 40 μL distilled water instead of experimental and standard materials. The control sample was measured daily. ABTS radical scavenging determination was applied to trolox solutions prepared at different concentrations (0.2-1 mM). The results of this study were given as mM trolox/mg extract [9].

2.6.3. Ferric Reducing/Antioxidant Power (FRAP) Assay

The method of Benzie and Strain (1996) was applied to the extracts/active compounds in order to estimate the ferric reducing ability. The FRAP reagent [25 mL 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution and 2.5 mL 20 mM FeCl_3·6H_2O] was kept at 37°C for 30 min. 190 μL FRAP reagent was mixed with 10 μL extract and the mixture absorbance was determined at 593 nm after 4 min. FRAP values of the extracts were given as mM Fe^{2+}/mg extract [10].

2.6.4. Cupric Ion Reducing/Antioxidant Power (CUPRAC) Assay

Briefly, 60 μL Cu(NO_3)_2·3H_2O, 60 μL neocuproine and 60 μL NH_4Ac (1 M) were mixed. Then, 60 μL of the extracts/active compounds and 10 μL of ethanol were added to the mixture. After the duration time of 60 min, the mixture absorbance was spectrophotometrically measured at 450 nm. CUPRAC values of the extracts were given as mM trolox/mg extract [11].

2.6.5. Total Phenolic Contents

Total phenolic content of plant extracts was determined with the Folin-Ciocalteu reagent (FCR) method. Briefly, 0.1 mL of the extract (5-0.5 mg/mL) was put in a plate and 4.5 mL of water was added. Then, 0.1 mL of the Folin-Ciocalteu reagent (diluted 1:3 with distilled water) and 0.3 mL of 2% sodium carbonate solution were added to the mixture. The mixture was left at room temperature for 3 min, and then absorbance was measured against the reference at 760 nm. Total phenolic contents were expressed as mg of gallic acid equivalents per mg of the extract [12].

2.7. Anti-urease Activity Assay

Briefly, working solution (100 μL) was taken and then urease (500 μL) was added on it. The mixture was incubated at 37°C for 30 min. Then, 1100 μL of urea was added to this mixture and kept in the incubator at 37°C for 30 min. R1 (1% phenol, 0.005% sodium nitroprusside) and R2 (0.5% NaOH, 0.1% sodium hypochlorite) reagents were added to the mixture, respectively. After the incubation period at 37°C for 2 h, the absorbance of samples was measured at 635 nm [13].

2.8. Anticholinesterase Activity Assay

Briefly, AChE (20 μL) and different concentrations of extracts (20 μL) were added to phosphate buffer solution (pH 8.20, 0.1 M, 40 μL). This mixture was incubated at 25°C for 10 min. After incubation, DTNB (100 μL) and AcI (20 μL) as substrate were added to the mixture. The same procedure was applied to the galantamine used as standard. The 5-thio-2-nitrobenzoic acid was spectrophotometrically measured at 412 nm [14].

2.9. Antimicrobial Activity

The antimicrobial activities of the extracts and compounds were examined by microbroth dilution method against *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida guilliermondii* KUEN 998, *Candida tropicalis* KUEN 1021, *Candida parapsilosis* ATCC 90018 and *Candida krusei* ATCC 6258 [15, 16].

2.10. Statistical Analysis

All the experiments were performed in triplicates. All data from the study were demonstrated as mean ± SD and analysed by the Graphpad Prism 5. Statistical differences between the study groups were analysed using two-way analysis of variance (ANOVA) followed by Tukey’s Multiple Comparison Test. Mean values were considered statistically significant when p<0.05.

3. RESULTS

3.1. Cytotoxic Activity of Different Extracts and Active Compounds from Plant

The cytotoxic activities of the n-hexan, chloroform, ethyl acetate and methanol extracts and active compounds on breast adenocarcinoma cell (MCF-7), breast adenocarcinoma cell receptor triple negative (MDA-MB-231) and healthy breast cell...
(184A1) were examined and results given in Table 1. According to the results obtained from this study, it was determined that chloroform extract showed strong and selective cytotoxic activity against MDA-MB-231 (IC_{50}: 7.00 mg/mL) and MCF-7 (IC_{50}: 8.63 mg/mL) cell lines. It was also found that the methanol extract had low activity against MCF-7 (IC_{50}: 25.32 mg/mL) and MDA-MB-231 (IC_{50}: 20.30 mg/mL) cell lines. When the results of the extracts on the normal cell line (184A1) were evaluated, the cytotoxic activity of the chloroform extract on cancer cells (IC_{50}: 7 mg/mL, 8.63 mg/mL) was found to be higher than in the normal cell line (IC_{50}: 15.82). Therefore, cytotoxic activity-guided isolation was made from chloroform extract and luteolin, naringenin and 8-hydroxy-salvigenin were isolated as the active components. When cytotoxic activities of active compounds were evaluated, it was determined that all compounds (especially naringenin compound) showed stronger and selective cytotoxic activity on cancer cells (MCF-7, MDA-MB-231) than normal cell. Therefore, it is contemplated that these compounds may be used in the future as cytotoxic agents.

Table 1. The cytotoxic activities of different extracts and active compounds from plant.

| Extracts       | Inhibition (IC_{50}: mg/mL) |
|----------------|-----------------------------|
| n-Hexan        | MCF-7 14.73±0.06; Chloroform 8.63±0.03; Ethyl acetate 18.93±0.08; Methanol 25.32±0.34; Luteolin 2.13±0.09; 8-hydroxy-salvigenin 0.64±0.05; Naringenin 0.57±0.23 |
| n-Hexan        | MDA-MB-231 184A1 8.63±0.03; Chloroform 7.00±0.04; Ethyl acetate 10.73±0.09; Methanol 20.30±0.05; Luteolin 2.41±0.06; 8-hydroxy-salvigenin 0.77±0.08; Naringenin 0.73±0.12 |

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts are significantly different, p<0.05.

3.2. Structure Elucidation of the Active Components

The structures of the isolates were elucidated on the basis of their 1H NMR and HR-MS analysis and by comparison of their spectroscopic data with those published earlier.

3.2.1. 8-hydroxy-salvigenin

It was obtained as a yellow amorphous powder; C_{18}H_{17}O_{3}, UV (MeOH) \( \lambda \text{max} \) : 214, 271, 336. IR (MeOH) \( \nu \text{max} \) cm^{-1}: 3316 (OH), 2943 (C=H), 1608 (C=O), 1441 (C-H). It was determined that all compounds (especially naringenin compound) showed stronger and selective cytotoxic activity on cancer cells (MCF-7, MDA-MB-231) than normal cell. Therefore, it is contemplated that these compounds may be used in the future as cytotoxic agents.

Table 2. The antioxidant activities and total phenolic contents of plant

| Samples          | DPPH (IC_{50}: mg/mL) | ABTS (mM trolox/mg extract) | FRAP assay (mM Fe^{3+}/mg extract) | CUPRAC assay (mM trolox/mg extract) | Total phenolic (mgGAE/mg extract) |
|------------------|-----------------------|-----------------------------|-----------------------------------|-----------------------------------|----------------------------------|
| n-hexan          | NA                    | 0.005±0.003*                | NA                                | 0.51±0.01*                        | 0.01±0.01*                        |
| Chloroform       | 0.90±0.16^             | 0.025±0.007*                | 0.167±0.009*                      | 1.03±0.03*                        | 0.002±0.001*                      |
| Ethyl acetate    | 0.18±0.001^            | 0.042±0.001*                | 0.054±0.015*                      | 2.38±0.05*                        | 0.049±0.0054*                     |
| Methanol         | 0.06±0.003*            | 0.067±0.003*                | 0.09±0.035*                       | 4.03±0.06*                        | 0.11±0.005*                       |
| Luteolin         | 0.07±0.002*            |                            | 0.77±0.54*                        | -                                 | -                                |
| 8-hydroxy-salvigenin | 0.91±0.048*           |                            | 3.48±0.94*                        | -                                 | -                                |
| Naringenin       | 0.151±0.029*           |                            | 8.73±0.99*                        | -                                 | -                                |
| Ascorbic acid    | 0.004±0.09^            | 1.3±0.1^                    | -                                 | 5.70±0.20^                        | -                                |
| BHT              | -                     | 1.1±0.12^                   | -                                 | -                                 | -                                |
| BHA              | 0.006±0.6^             | -                           | -                                 | -                                 | -                                |

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts are significantly different, p<0.05; NA: not activity.

3.3. Biological Activities of Isolated Compounds and Different Extracts from Plant

3.3.1. Total Phenolic Contents

The total phenolic contents of different extracts were analyzed and presented in Table 2. When we compared the results obtained, it was found that methanol extract had the highest amount of phenolic contents.
3.3.2. The Antioxidant Activity

The methanol extract from plant showed stronger DPPH free radical scavenging (IC$_{50}$: 0.06 mg/mL), ABTS radical cation scavenging (0.067 mM trolox/mg extract) and cupric reducing antioxidant (4.03 mM trolox/mg extract) activity than other extracts. In addition, chloroform extract exhibited the highest ferric reducing activity (0.167 mM Fe$^{2+}$/mg extract). In this study, it was found that n-hexan extract showed no activity in the two methods (DPPH, FRAP) and the lowest activity in the other two methods (ABTS, CUPRAC). As shown in Table 2, the antioxidant activities of all extracts were lower than that of standard compounds (ascorbic acid, BHT, BHA). The antioxidant activities of the compounds isolated from the plant were examined using ABTS and FRAP methods and naringenin was found to have the strongest antioxidant activity. It was also determined that the naringenin and 8-hydroxy-salvigenin compounds had stronger ferric reducing activity than the standard BHT. When antioxidant activities of extracts and isolated compounds were compared, it was determined that isolated compounds had stronger ABTS radical cation scavenging and ferric reducing activity than extracts.

3.3.3. Urease and Acetylcholinesterase Inhibitory Activity

The results of anti-urease and anticholinesterase activity of different extracts and active compounds are shown in Table 3. The n-hexan extract (IC$_{50}$: 0.023 mg/mL) exhibited the strongest anti-urease activity. In this study, it was also found that chloroform (IC$_{50}$: 0.031 mg/mL) and ethyl acetate extracts (IC$_{50}$: 0.030 mg/mL) had a very close anti-urease activity. Besides, the anti-urease activities of all extracts was shown to be lower than that of the standard Thiourea. When the anticholinesterase activities of plant extracts were compared, it was found that methanol extract (IC$_{50}$: 0.018 mg/mL) had activity close to the standard compound (IC$_{50}$: 0.012 mg/mL) and strong anticholinesterase activity than other extracts. When the activities of the isolated compounds on enzyme inhibition were evaluated, it was found that the 8-hydroxy-salvigenin compound had strong inhibition activity on both urease (IC$_{50}$: 0.043 mg/mL) and acetylcholinesterase (IC$_{50}$: 0.01 mg/mL) enzymes.

| Samples               | Urease inhibition (IC$_{50}$, mg/mL) | Acetylcholinesterase inhibition (IC$_{50}$, mg/mL) |
|-----------------------|-------------------------------------|----------------------------------------------------|
| n-hexan               | 0.023±0.004$^a$                     | 0.034±0.002$^a$                                   |
| Chloroform            | 0.031±0.004$^a$                     | 0.031±0.004$^a$                                   |
| Ethyl acetate         | 0.030±0.005$^b$                     | 0.027±0.001$^b$                                   |
| Methanol              | 0.090±0.071$^a$                     | 0.018±0.011$^a$                                   |
| Luteolin              | 1.20±0.39$^a$                       | 0.014±0.002$^a$                                   |
| 8-hydroxy-salvigenin  | 0.043±0.004$^a$                     | 0.01±0.0001$^a$                                   |
| Naringenin            | 0.083±0.049$^b$                     | 0.033±0.006$^b$                                   |
| Thiourea              | 0.002±0.001$^a$                     | -                                                  |
| Galantamine           | -                                   | 0.012±0.001$^a$                                   |

Values are mean of triplicate determination (n = 3) ± standard deviation; Means with different superscripts (a,b) are significantly different, p<0.05

3.3.4. Antimicrobial Activity

In this study, the ethyl acetate extract showed antifungal activity against C. glabrata, C. krusei and C. parapsilosis while it did not show against bacteria. Similarly, the n-hexan extract showed antifungal activity against C. glabrata, C. guilliermondii, C. tropicalis, C. krusei and C. parapsilosis. In contrast, we did not find any effect on bacteria. The methanol extract showed antifungal activity against C. glabrata and C. krusei. The chloroform extract showed antibacterial activity against S.aureus and antifungal activity against all standard Candida species. The luteolin compound showed antifungal activity against C. albicans, C. guilliermondii, C. tropicalis, C.krusei and C. parapsilosis. The naringenin compound showed antibacterial activity against P. vulgaris, P. aeruginosa and antifungal activity against Candida species except C. glabrata. The 8-hydroxy-salvigenin compound showed antibacterial activity against P. vulgaris, P. aeruginosa, S. epidermidis and E. coli and antifungal activity against C. albicans, C.glabrata, C. guilliermondii, C.krusei and C. parapsilosis (Table 4).
A literature survey revealed that there are only two reports regarding the chemical contents and biological activity of *A. monocephala*. The contents of the essential oil obtained from the leaves and flowers of the plant were examined, borneol in the leaves and camphor, borneol, 1,8-cineole in the flowers were found to be major compounds [25, 26]. In another study, the biological activities of ethanol extracts from root and aerial parts of the plant were investigated. In this study, it was observed that the root extract exhibited strong antioxidant and the aerial extract strong anticholinesterase and anti-urease activities. The chloroform: methanol (1:1, v/v) extract from plant’s root exhibited strong cytotoxic activity against HeLa cell line. In addition, quinic acid, malic acid, fumaric acid, chlorogenic acid, vanillic acid, rutin, luteolin and apigenin compounds as major from root and aerial parts were analyzed using LC-MS/MS [27]. In the present study, different from the above studies, biological activities (antioxidant, cytotoxic, anti-urease, anticholinesterase, antimicrobial) of different extracts (n-hexane, chloroform, ethyl acetate and methanol) from aerial parts of the plants were investigated. The active compounds (luteolin, 8-hydroxy-salvigenin, naringenin) were then isolated from the chloroform extract showing strong cytotoxic activity. Finally, the biological activities (antioxidant, cytotoxic, anti-urease, anticholinesterase, antimicrobial) of the isolated compounds were determined and the relationship between the active compounds and the biological activity was determined. The biological activity of luteolin and naringenin compounds isolated from the plant has been previously reported in several studies. The antioxidant (DPPH, linoleic acid peroxidation, xanthine oxidase), antimicrobial and cytotoxic (SGC-7901, ABT-737 and HeLa cell lines) activities [35 - 39]. When we compare the results of naringenin compound with the literature, it was observed that we obtained parallel results. In addition, naringenin compound was found to have potent anti-urease and anticholinesterase activities in this study.

**CONCLUSION**

The results of this study clearly demonstrated that the plant has potent antioxidant, cytotoxic, anti-urease, anticholinesterase and antimicrobial activities. The methanol extract was found to exert significant *in vitro* antioxidant and anticholinesterase activity. The chloroform extract showed the strongest cytotoxic and antimicrobial activity. The active components in chloroform were determined to be luteolin, naringenin, 8-hydroxy-salvigenin. The naringenin and 8-hydroxy-salvigenin compounds exhibited the strongest antioxidant, cytotoxic, antimicrobial, anti-urease and anticholinesterase activity.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

Not applicable.

**HUMAN AND ANIMAL RIGHTS**

No animal/human were used in this research.

**CONSENT FOR PUBLICATION**

Not applicable.

**AVAILABILITY OF DATA AND MATERIALS**

Not applicable.

**FUNDING**

None.
Cytotoxic Activity-Guided Isolation

CONFLICT OF INTEREST
The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS
Declared none.

REFERENCES
[1] Hossein MM, Sarker SD, Akbarzadeh A. Chemical composition of theessential oils and extracts of Achillea species and their biological activities: a review. J Ethnopharmacol 2017; 199: 315-20. [PMID: 28261565]
[2] Sacidnia S, Gohari A, Mohkber-Dezfuli N, Kiouchi F. A review on phytochemistry and medicinal properties of the genus Achillea. Daru 2011; 19(3): 173-86. [PMID: 22615655]
[3] Kăpilei E, Orhan I, Küsmenoglu S, Yesildaş E. Evaluation of Antiinflammatory and antinociceptive activity of five anatolian Achillea species. Turk J Pharm Sci 2007; 4: 99-102.
[4] Greenwell M, Rahman PKSM. Medicinal Plants: Their use in antitumor treatment. Int J Pharm Sci Res 2015; 6(10): 4101-12. [PMID: 26594645]
[5] Pejin B, Talevska D, Rayaman E. In vitro and In vivo CUPRAC method. J Agric Food Chem 2004; 52(26): 7970-81.
[6] Taskin T, Çam ME, Taşkın D, Rayaman E. Bioassay-guided isolation and identification of antioxidant flavonoids from Cyclotrichium argiophorum (Labill.) and Manden and Scheng. Pharmacogn Mag 2017; 13(50): 316-20. [PMID: http://dx.doi.org/10.4103/0973-1296.204556] [PMID: 28539727]
[7] Jain R, Mittal M, Narangenn A. Flavonone from the stem of Nyctanthes arbor-tristis Linn. JBPAS 2012; 1: 964-9.
[8] Cordenonsi LM, Sponchiado RM, Campanaro SC, Garcia CV, Raffin RP, Schapoval EES. Study of flavonoids presente in pomelo (Citrus maxima) by DSC, UV-VIS, IR, HR AND 13C NMR and MS. Drug Anal Res 2017; 1: 31-6. [http://dx.doi.org/10.22456/2527-2616.74097] [PMID: 28620306]
[9] Nile SH, Keum YS, Nile AS, Jalil SS, Patel RV. Antioxidant, anti-inflammatory, and enzyme inhibitory activity of natural plant flavonoids and their synthesized derivatives. J Biochem Mol Toxicol 2018; 32(1): 22-8. [http://dx.doi.org/10.1002/jbm.22002] [PMID: 28972678]
[10] Leung HWC, Kuo CL, Yang WH, Lin CH, Lee HZ. Antioxidant enzymes activity involvement in luteolin-induced human lung squamous carcinoma CH27 cell apoptosis. Eur J Pharmocol 2006; 534(1-3): 12-8. [http://dx.doi.org/10.1016/j.ejphar.2006.01.021] [PMID: 16469309]
[11] Garcia OB, Castillo J, Lorente J, Ortun AA, Rio JA. Antioxidant activity of phenolics extracted from Olea europaea L. Leaves. Food Chem 2000; 68: 457-61. [http://dx.doi.org/10.1016/S0927-6512(00)00221-6] [PMID: 10935355]
[12] Mota KSL, Dias GEN, Pinto ME, Waz RP, Schapoval EES. Study of flavonoids presente in pomelo (Citrus maxima) by DSC, UV-VIS, IR, HR AND 13C NMR and MS. Drug Anal Res 2017; 1: 31-6. [http://dx.doi.org/10.22456/2527-2616.74097] [PMID: 28620306]
[13] Hassan STS, Zemlička M. Plant-derived urinary inhibitors as alternative chemotherapeutic agents. Arch Pharm (Weinheim) 2016; 349(7): 507-22. [http://dx.doi.org/10.1002/ardp.201500019] [PMID: 27244041]
[14] Yoo DR, Jang YH, Jeon YK, et al. Proteomic identification of anti-cancer proteins in luteolin-treated human hepatoma Hep-7 cells. Cancer Lett 2009; 282(1): 48-54. [http://dx.doi.org/10.1016/j.canlet.2009.02.051] [PMID: 19342156]
[15] Furad IM, Hussein SR, Ibrahim LF, et al. Cytotoxic activity and phytochemical analysis of Arum palaestinum Boiss. Asian Pac J Trop Biomed 2015; 1: 1-9. [http://dx.doi.org/10.1016/j.ajphb.2015.07.019]
[16] Lewis AC. A. monocephala. In vitro and In vivo biological activities and phenolic characterization of Thymus praecox subsp. skarpetii var. skarpetii. J Food Meas Charact 2019; 13: 544-8. [http://dx.doi.org/10.1002/ffj.1541] [PMID: 29602084]
[17] Gogus F, Ozel MZ, Lewis AC. Extraction of essential oils of leaves and flowers of Achillea. Daru 2011; 19(3): 173-86. [PMID: 22615655]
[18] Intekhab I, Aslam M. Isolation of a flavonoid from the roots of Citrus sinensis. Malays J Pharm Sci 2006; 7: 8-12.
[19] Iqbal K, Iqbal J, Stærk D, Kongstad KT. Characterization of antileishmanial compounds from Lawsonia inermis L. Leaves using semi-high resolution antileishmanial profiling combined with HPLC-HRMS-SPE-NMR. Front Pharmacol 2017; 8: 337. [http://dx.doi.org/10.3389/fphar.2017.00337] [PMID: 28620306]
[20] Tahira AW, Begüm S, Ayab A, et al. Luteolin and kaempferol from Cassia alata, antimicrobial and antioxidant activity of its methanolic extracts. FUAST J Biology 2014; 4: 5-9.
[21] Rahate KP, Rajasekar A. Isolation and Identification of flavone aglycones in roots of Desmostachya bipinnata Stapf. Indian J Pharm Sci 2018; 80: 556-9. [http://dx.doi.org/10.4172/pharmaceutical-sciences.1000391] [PMID: 29602084]
[37] Kozłowska J, Potaniec B, Zarowska B, Aniol M. Synthesis and biological activity of novel α-alkyl derivatives of naringenin and their oximes. Molecules 2017; 22(9): 1485-92.
[38] Zhang H, Zhong X, Zhang X, Shang D, Zhou YI, Zhang C. Enhanced anticancer effect of ABT-737 in combination with naringenin on gastric cancer cells. Exp Ther Med 2016; 11(2): 669-73.
[39] Krishnakumar N, Sulfikkarali N, Prasad NR, Karthikeyan S. Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (HeLa) cancer cells. Biomedicine & Preventive Nutrition 2011; 1: 223-8.