

RESEARCH ARTICLE

Antioxidant and Cytotoxic Activities of *Achillea millefolium* from Kashmir

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Abstract

Antioxidant and cytotoxic activities of *Achillea millefolium* were investigated in this study. The cytotoxic effect of methanol and chloroform extracts of *A. millefolium* against four human cancer cell lines: breast (MCF-7), leukemia (THP-1), prostate (PC-3) and ovarian (OVCAR-5) was evaluated using Sulpharhodamine B (SRB) assay. The antioxidant activity of the extracts was determined using two assay systems: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays and that of chemical constituents was evaluated using DPPH assay. All the extracts exhibited broad spectrum cytotoxicity against all the four investigated cancer cell lines with chloroform extract of aerial part of *A. millefolium* (AMRC) showing growth inhibition of 99%, 98% and 99% against THP, MCF-7 and OVCAR-5 cell lines at 50 µg/mL concentration. All the six extracts and isolated constituents were screened for antioxidant activity and exhibited dose dependent antioxidant activity. AMRC, quercetin and dihydroquercetin showed the best results with IC₅₀ of 2.5, 2.2 and 2.26 µg/mL respectively.

Keywords: Antioxidant, cytotoxicity, *Achillea millefolium*, DPPH, human cancer cell lines, AMRC.

Introduction

The genus *Achillea* is widespread all over the world and many species of this genus have been used as traditional herbal medicine. *Achillea millefolium* (Asteraceae), commonly known as Yarrow is a herbaceous flowering plant native to temperate regions of the northern hemisphere in Asia, Europe and North America (Simonetti, 1990). The cytotoxic effects of extracts of *Achillea alexandri-regis*, *A. clavennae* and *A. millefolium* have been reported against various malignant tumour cell lines (Csupor-Loffler *et al.*, 2009). Extracts of *A. millefolium* are used in the treatment of gastrointestinal disorders, skin inflammation and wound healing (Benedek *et al.*, 2007a). Yarrow is a good bio-enhancer and thereby intensifies the therapeutic effects of other herbs taken with it (Benedek *et al.*, 2006). It is reported to be associated with the treatment of the ailments *viz-a-viz* pain (Noureddini and Rasta, 2008), antiphlogistic (Benedek *et al.*, 2007b; Burk *et al.*, 2010), gastrointestinal disorders (Noureddini and Rasta, 2008), inflammation (Popovici *et al.*, 2008). In India, the leaves and flowers of *A. millefolium* are used for gastric problems and fever (Sharma *et al.*, 2004). In Iran, the infusion of dried flowers is considered suitable for the treatment of hemorrhoids, dyspepsia and gastritis (Miraldi *et al.*, 2001). The formation of reactive oxygen species (ROS) is characteristic of aerobic organisms that normally defend themselves against the highly reactive species using enzymes (like peroxidases and superoxide dismutases) and naturally occurring antioxidants

(Burguete *et al.*, 2007). ROS like superoxide radical anion, hydrogen peroxide radical etc. are produced during the inflammation process by phagocytic leucocytes (neutrophils, monocytes and eosinophils) that invade the tissue. Persistently high levels of ROS may result into pathological conditions as these reactive species can modify the biomolecules like proteins, lipids and DNA. The above pathophysiological conditions could be treated by the use of antioxidants (Burguete *et al.*, 2007).

As part of our research work on the bioprospection of medicinal plants growing in Kashmir, *Achillea millefolium* was selected for bioactivity guided phytochemical investigation. Herein, we report the antioxidant and cytotoxic activities of *Achillea millefolium* and the isolation of chemical constituents from the most potent root extract (AMRC).

Materials and methods

Plant material extraction: *Achillea millefolium* was collected in the month of June from Institute's field station, Bonera, Kashmir (India). Floral, aerial and root parts of the plant were separated, shade dried for 3 days and grinded to powder. The powdered plant material was separately extracted with chloroform and methanol using cold extraction method and the filtrate obtained was concentrated under vacuum using Buchi rotary evaporator to get two crude extracts (AMRC and AMRM).

Isolation of chemical constituents from AMRC extract: About 65 g of methanol extract of *Achillea millefolium* root were subjected to repeated column chromatography over silica gel to yield six constituents: Quercetin (27 mg), Dihydroquercetin (33 mg), Chlorogenic-acid (12 mg), Apigenin-7-O-glucoside (40 mg), Luteolin-7-O-glucoside (23 mg) and Salvigenin (15 mg) using hexane-ethylacetate as eluent with increasing polarity of 5%, 10%, 20%, 35%, 45% and finally 65% ethyl acetate.

Chlorogenic acid: White amorphous powder; mp. 206-210°C; ¹H-NMR (500 MHz, CD₃OD₂): 2.00 (H-1, dd, J = 13.8, 9.0 Hz, H-2ax), 2.02 (1H, dd, J = 13.8, 4.0 Hz, H-6eq), 2.09 (1H, dd, J = 3.68), 3.4 Hz, H-6ax) 2.13 (1H, dd, J = 13.8, 4.5 Hz, H-2eq), 3.68 (1H, dd, J = 8.7, 3.3, H-4), 4.12 (1H, m, H-5), 5.28 (1H, ddd, J = 9.0, 8.7, 4.5 Hz, H-3), 6.12 and 7.51 (each 1H, d, J = 15.9 Hz, H-α, β), 6.74 (1H, d, J = 8.0 Hz, H-5'), 6.90 (1H, dd, J = 8.0, 2.1 Hz, H-6'), 7.01 (1H, d, J = 2.1 Hz, H-2'); ¹³C-NMR (125 MHz, CD₃OD₂): 76.3 (C-1), 38.2 (C-2), 72.0 (C-3), 73.6 (C-4), 71.4 (C-5), 38.9 (C-6), 76.3 (C-7), 115.2 (C-α), 147.1 (C-β), 168.9 (C-9), 127.8 (C-1'), 115.2 (C-2'), 146.6 (C-3'), 149.4 (C-4'), 116.5 (C-5'), 123 (C-6'), 177.5 (C-7').

Quercetin: Slightly yellow powder; mp. 312-316°C; ¹H-NMR (500 MHz, MeOD): δ (ppm) = 6.18 (1H, d, J = 2.0 Hz, H-6), 6.39 (1H, d, J = 2.0 Hz, H-8), 6.88 (1H, d, J = 8.3 Hz, H-5'), 7.62 (1H, dd, J = 8.3; 2.1 Hz, H-6'), 7.74 (1H, d, J = 2.1 Hz, H-2'), ¹³C-NMR (125MHz, MeOD): δ (ppm) = 94.6 (CH, C-8), 99.4 (CH, C-6), 104.7 (C, C-10), 116.1 (CH, C-2', C-5'), 121.8 (CH, C-6'), 124.3 (C, C-1'), 137.2 (C, C-3), 146.3 (C, C-3'), 148.2 (C, C-2), 150.3 (C, C-4'), 158.4 (C, C-9), 162.6 (C, C-5), 165.7 (C, C-7), 177.5 (C, C-4).

Apigenin-7-O-glycoside: Yellow crystals; mp. 230-237°C; ¹H-NMR (400 MHz DMSO d₆): δ 7.94 (2H, d, J = 8.4Hz, H-2',6'), 6.94(2H, d, J = 8.4Hz, H-3',5'), 6.87 (1H, s, H-3), 6.83 (1H, bs, H-8), 6.43 (1H, bs, H-6), 5.08(1H, d, J = 6.8 Hz, Glu H-1'), 3.25-5.08(6H, m, sugar protons); ¹³C-NMR (DMSO d₆): δ 181.7(C-4), 164.0 (C-2), 162.3 (C-7), 161.3 (C-4'), 160.9 (C-5), 156.8 (C-9), 128.4 (C-2',6'), 120.7 (C-1'), 115.9 (C-3',5'), 105.3 (C-10), 103.0 (C-3), 99.2 (C-6), 94.4 (C-8) and sugar: 97.7 (C-1''), 77.1 (C-5''), 76.9 (C-3''), 76.2 (C-2''), 69.6 (C-4''), 60.4 (C-6'').

Luteolin-7-O-glycoside: Yellow amorphous powder; mp. 266-268°C; ¹H-NMR (DMSO d₆): δ 7.42 (1H, bs, H-2'), 7.39(1H, bd, J = 8.4 Hz, H-6'), 6.92(1H, d, J = 8.4 Hz, H-5'), 6.81(1H, d, J = 2 Hz, H-8), 6.73(1H, s, H-3), 6.44(1H, d, J = 2 Hz, H-6), 5.05(1H, d, J = 6.6 Hz, Glu H-1'), 3.5-5.05(6H, m, sugar protons); ¹³C-NMR (DMSO d₆): δ 182.1(C-4), 163.8 (C-2), 162.8 (C-7), 161.2 (C-5), 157.0 (C-9), 149.8 (C-4'), 145.8 (C-3'), 121.4 (C-1'), 120.2 (C-6'), 116.0 (C-5'),

113.5 (C-2'), 105.1 (C-10), 103.2 (C-3), 99.6 (C-6) 95.0 (C-8) and sugar: 98.0 (C-1''), 78.4 (C-2''), 77.1 (C-5''), 75.8 (C-3''), 69.6 (C-4''), 60.6 (C-6'').

Salvigenin: Yellow coloured mp. 186-190°C; ¹H NMR (CDCl₃, 400 MHz) δ: 12.7 (1H, s), 7.84 (1H, d, J = 8.8 Hz), 7.02 (1H, d, J = 8.8 Hz), 6.54 (1H, s), 6.58 (1H, s), 3.97, 3.92, 3.89 (each 3H, s); ¹³C-NMR (CDCl₃, 125 MHz) δ: 183.1, 164.3, 162.9, 159, 153.5, 127.9, 123.8, 114.5, 104.1, 90.5, 60.8, 56.3, 53.4.

Dihydroquercetin: Pale yellow needles; mp. 222-224°C; [α]_D²⁰ +42.5 (c 1.30, acetone); ¹H-NMR (400 MHz, MeOD) δ: 6.95 (1H, d, J = 1.2 Hz, H-2'), 6.83 (1H, dd, J = 8.0, 1.6Hz, H-6'), 6.79 (1H, d, J = 8.0 Hz, H-5'), 5.87 (2H, s, H-6, 8), 4.90 (1H, d, J = 11.6 Hz, H-2), 4.49 (1H, d, J = 11.5 Hz, H-3); ¹³C-NMR(100 MHz, MeOD)δ: 198.4 (C-4), 168.8 (C-7), 165.3 (C-5), 164.5 (C-9), 147.1 (C-4'), 146.3 (C-3'), 129.9 (C-1'), 120.9 (C-6'), 116.1 (C-2'), 115.9 (C-5'), 101.8 (C-10), 97.3 (C-6), 96.3 (C-8), 85.1 (C-2), 73.7 (C-3).

Chemicals: RPMI-1640 medium, Streptomycin, Fetal bovine serum, Mitomycin, Sodium bicarbonate, Phosphate buffer saline (PBS), Sulphorhodamine (SRB), Trypsin, Paclitaxel, 5-Fluorouracil (5-FU), Doxorubicin and Gentamycin sulphate were purchased from sigma chemical Co.; Tris buffer was procured from Himedia. Glacial acetic acid was purchased from Fisher Scientific; Chloroform, Methanol and Trichloroacetic acid (TCA) from Merck Specialties Pvt. Ltd. All the chemicals used in the present study were of molecular biology grade. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), L-ascorbic acid, were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals/solvents were of analytical grade.

Cell culture, growth conditions and treatment: The human cancer cell lines: Human breast cancer cell line (MCF-7), human acute monocytic leukemia cell line (THP-1), human prostate cancer cell line (PC-3) and human ovarian cancer cell line (OVCAR-5) were obtained from ATCC sigma. The cells were grown in RPMI-1640/ MEM medium containing 10% FCS, 100 unit Penicillin/100 µg Streptomycin per mL medium. Cells were allowed to grow in CO₂ incubator (Thermo Scientific USA) at 37°C with 98% humidity and 5% CO₂ gas environment.

Cytotoxic activity: The various extracts were subjected to screening for the possible cytotoxic potential by using Sulphorhodamine B assay.

Sulpharhodamine B assay for % growth inhibition: In the present study the cytotoxic effect of different extracts were evaluated using Sulpharhodamine B (SRB) assay.

Table1. Cytotoxicity of methanol and chloroform extracts of different parts of *Achillea millefolium*.

| Code | Conc. (µg/mL) | Percentage inhibition | | | |
|-------------|---------------|-----------------------|---------------|--------------|---------------|
| | | Leukemia/THP | Prostate/PC-3 | Breast/MCF-7 | Ovary/OVCAR-5 |
| AMAM | 50 | 58 ± 1.7 | 20 ± 2.5 | 46 ± 2.3 | 19 ± 1.8 |
| AMAC | 50 | 78 ± 1.3 | 40 ± 2.7 | 75 ± 2.4 | 49 ± 1.6 |
| AMFM | 50 | 67 ± 2.3 | 31 ± 1.9 | 70 ± 2.1 | 46 ± 1.7 |
| AMFC | 50 | 99 ± 0.9 | 77 ± 2.1 | 98 ± 1.9 | 99 ± 0.7 |
| AMRM | 50 | 51 ± 1.9 | 33 ± 1.9 | 19 ± 1.9 | 27 ± 2.4 |
| AMRC | 50 | 98 ± 1.3 | 38 ± 2.4 | 15 ± 2.6 | 57 ± 2.4 |
| 5-FU | 50 | 88 ± 1.6 | 66 ± 2.5 | - | - |
| Adriyamycin | 50 | - | - | 76 ± 1.9 | - |

AM= *Achillea millefolium*, A=Aerial/shoot part, F=Floral part, R= Root part, C=Chloroform, M=Methanol, FU=Fluoro uracil.

The SRB dye binds to the basic protein of cells that have been fixed to tissue-culture plates by TCA. As the binding of SRB is stoichiometric, the amount of dye extracted from stained cells is directly proportional to the cell number. In the present case, all cells lines seeded in flat-bottomed 96-well plates were allowed to adhere overnight and then media containing different plant extracts were added. The plates were assayed for 48 h. The cells were fixed by adding 50 µL of ice-cold 50% TCA to each well for 60 min. The plates were washed five times in running tap water and stained with 100 µL per well SRB reagent (0.4% w/v SRB in 1.0% acetic acid for 30 min). The plates were washed five times in 1.0% acetic acid to remove unbound SRB and allowed to dry overnight. SRB was solubilized with 100 µL per well 10 mM Tris-base, shaken for 5 min and the OD was measured at 570 nm with reference wavelength of 620 nm.

Antioxidant activity: The extracts and compounds isolated were subjected to screening for possible antioxidant activity by two methods i.e. DPPH and ABTS free radical scavenging assays.

DPPH free radical-scavenging activity: DPPH free radical scavenging activity was evaluated by measuring the scavenging activity of the test samples against stable 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH). A 0.5mM solution of DPPH in methanol was prepared and a stock solution of extracts and compounds isolated (1.0 mg/mL) in methanol was prepared. Various concentrations of test samples were added to 1.0 mL of 0.5mM DPPH solution and final volume was made to 3.0 mL with methanol to get the desired concentrations. The mixture was shaken thoroughly and kept standing at room temperature for 10 min. Then the absorbance of the mixture was measured at 517 nm on spectrophotometer. The decrease in the absorbance indicates an increase in DPPH-radical scavenging activity. The percentage inhibition was calculated by the following equation.

$$\text{DPPH radical scavenging (\%)} = \left\{ \frac{A_c - A_s}{A_c} \right\} \times 100$$

Where, A_c is the absorbance of control, A_s is absorbance of sample. L-ascorbic acid was used as positive control. The experiment was done in triplicate and mean values were recorded.

ABTS assay: The ABTS assay was employed to measure the antioxidant activity of the test material. This assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the reactive ABTS radical (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). Blue-green ABTS was produced through the reaction between 7 mM ABTS and 2.45 mM potassium per sulfate in water. The reaction mixture was left to stand at room temperature for 30 min in the dark. The resultant intensely colored ABTS was diluted with PBS, pH 7.4 to give an absorbance value of ~0.70 at 734 nm. Percentage antioxidant activity was calculated as:

$$\frac{(\text{OD Control} - \text{OD Test})}{(\text{OD Control})} \times 100$$

Discoloration of the ABTS dye was taken as an index of antioxidant potential. All the volumes were taken in triplets.

Statistical analysis: Each experiment was done in triplicate and mean values were calculated. The data were recorded as means ± standard deviations. Analysis of variance for individual parameters was performed on the basis of mean values to find out the significance at $p < 0.05$.

Results and discussion

Cytotoxicity: The cytotoxic effects of the extracts prepared from all the three plant parts separately (root, aerial and flower) of *Achillea millefolium* were evaluated against THP, PC-3, MCF-7 and OVCAR-5 cell lines using SRB assay system. The dried plant material of root, aerial and floral parts was separately subjected to extraction with methanol. The methanol extracts of all the three parts were subsequently partitioned with chloroform yielding chloroform and methanol soluble extracts. Both the chloroform and methanol extracts (AMAM, AMAC, AMFM, AFMC, AMRC and AMRM) were screened for cytotoxicity against a panel of four cell lines at 50 µg/mL concentration (Table 1). The chloroform extract of flowers exhibited noteworthy tumor cell growth inhibitory activity against Leukemia/THP, Brest/MCF-7 and Ovary/OVCAR-5 cell lines with percentage growth inhibition of 99, 98 and 99% respectively.

Fig. 1. Antioxidant activity of extracts using DPPH assay.

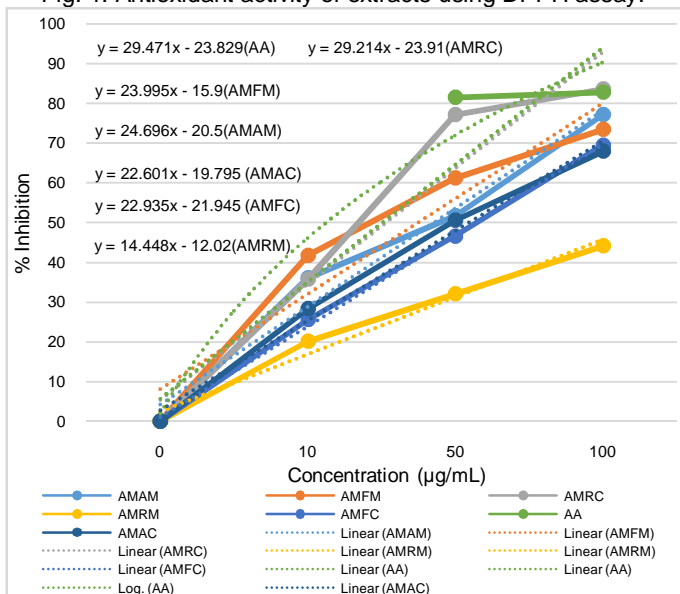
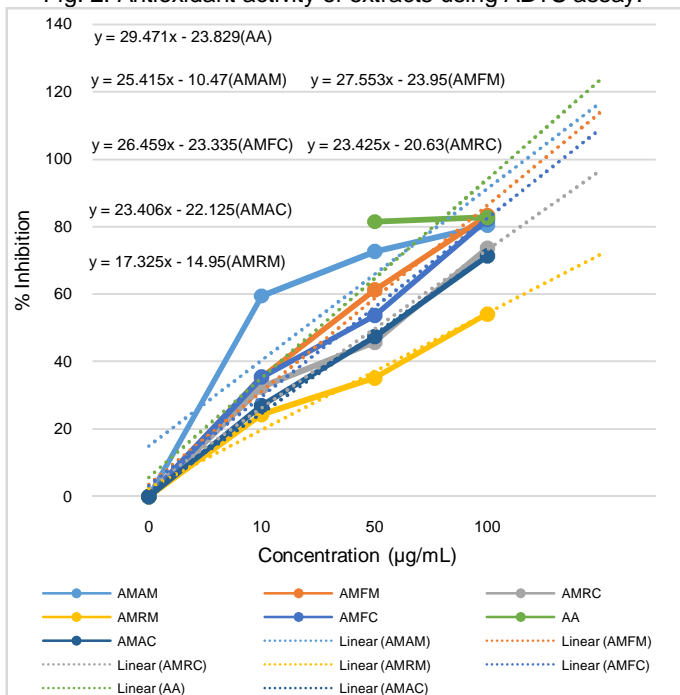


Fig. 2. Antioxidant activity of extracts using ABTS assay.



On the other hand, chloroform extract of roots (AMRC) showed selective cytotoxicity against leukemia cell line with 98% growth inhibition.

Antioxidant activity: In order to ascertain the antioxidant potential of *A. millefolium* extracts (AMAM, AMAC, AMFM, AMFC, AMRM and AMRC), two spectrophotometric methods (DPPH and ABTS) were employed (Fig. 1 and 2).

DPPH is the most widely used assay system for the determination of antioxidant activity as it offers a rapid technique for screening the radical scavenging ability of specific compounds, essential oils or extracts (Tahseen *et al.*, 2013). In this method, the antioxidants reduce the purple colored DPPH radical solution to yellow colored diphenylpicryl hydrazine. DPPH possesses a characteristic absorption at 517nm which decreases significantly on exposure to radical-scavengers (antioxidants) by providing hydrogen atom or electron donation. A lower absorbance at 517nm indicates a higher radical-scavenging activity of the sample under study. Both chloroform and methanol extracts of each plant part were subjected to DPPH radical scavenging activity against the reference standard ascorbic acid. The variation of % antioxidant activity with concentration of test samples is shown in Fig. 1.

The chloroform extract of root (AMRC) shows the highest DPPH radical scavenging activity with an IC_{50} of 2.5 $\mu\text{g/mL}$ comparable to that of reference standard ascorbic acid (IC_{50} of 2.5 $\mu\text{g/mL}$) (Table 2). On the other hand, methanol extract of the aerial part of *Achillea millefolium* (AMAM) exhibited a dose dependent highest oxidation potential of all the six extracts screened for antioxidant activity using ABTS assay system with an IC_{50} of 2.3 $\mu\text{g/mL}$ (Fig. 2). The chloroform extract of root was selected for a detailed phytochemical-pharmacological analysis so that the antioxidant constituents could be isolated and identified. Rigorous phytochemical analysis of chloroform extract of root afforded six compounds viz. chlorogenic acid, quercetin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, salvigenin and dihydroquercetin. The isolates were identified on the basis of comparison of NMR data (^1H and ^{13}C NMR) with that reported in literatures (Jassbi *et al.*, 2002; Yassa *et al.*, 2007; Ahmad, 2010; Mouffok *et al.*, 2012; Pereira *et al.*, 2013).

Among the isolated natural products, chlorogenic acid, apigenin-7-O-glucoside and luteolin-7-O-glucoside were previously reported from *Achillea millefolium* (Vitalini *et al.*, 2011). Salvigenin and quercetin are being reported for the first time from *Achillea millefolium* but simultaneously represent the main characteristic secondary metabolites of genus *Achillea* (Si *et al.*, 2006). Though quercetin and its derivatives like quercetin-3-methyl ether and quercetin 3, 3'-dimethyl ether exist in *Achillea* species (Si *et al.*, 2006) but dihydroquercetin has not been reported earlier from any *Achillea* species. The isolated compounds were screened for antioxidant activity using DPPH assay system (Table 2). All the isolates showed potent radical scavenging activity with IC_{50} value of 2.7, 2.2, 3.1, 2.2, 2.26 and 6.34 $\mu\text{g/mL}$ for chlorogenic acid, quercetin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, dihydroquercetin and salvigenin respectively.

Table 2. Antioxidant activity of methanol and chloroform extracts of *Achillea millefolium* and the isolated constituents.

| Compound/Extract | IC ₅₀ (DPPH assay) µg/mL | IC ₅₀ (ABTS assay) µg/mL |
|------------------------|-------------------------------------|-------------------------------------|
| AA (Ascorbic acid) | 2.5 | 2.5 |
| Chlorogenic acid | 2.7 | - |
| Quercetin | 2.2 | - |
| Apigenin 7-O-glucoside | 3.1 | - |
| Luteolin 7-O-glucoside | 2.2 | - |
| Dihydroquercetin | 2.26 | - |
| Salvigenin | 6.34 | - |
| AMAM | 2.8 | 2.3 |
| AMFM | 2.7 | 2.7 |
| AMRC | 2.5 | 3.01 |
| AMFC | 3.1 | 2.7 |
| AMRM | 4.2 | 3.7 |
| AMAC | 3.08 | 3.0 |

Conclusion

Achillea millefolium showed promising antioxidant and cytotoxic activities. AMRC extract possesses selective cytotoxic effects against Leukemia/THP cell line; AMRC, quercetin and dihydroquercetin showed the best radical scavenging activity with IC₅₀ of 2.5, 2.2 and 2.26 µg/mL respectively.

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