Original Article

Biological Evaluation of *Mercurialis annua* Extracts for Possible Antioxidant, Antiproliferative and Cytotoxic Activity

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ABSTRACT

Background: *Mercurialis annua* has been traditionally used by native people for its diuretic, laxative, antiemetic and antioxidant activity in addition to its usefulness in the treatment of warts, eye problems and microbial infections. **Aim:** The goal of this work is to study the different extracts of aerial parts of *Mercurialis annua* collected from the Jordan territories and test them for possible antioxidant, cytotoxic and antiangiogenic activities. **Materials and Methods:** One kilogram of the dried powdered aerial parts of *M. annua*, was extracted by maceration with n-hexane, ethyl acetate, and ethanol (96%), respectively. Flavonoids were detected and identified by UV spectrophotometer, TLC, NMR, Mass, and melting point in comparison with the standard. The brine-Shrimp cytotoxicity /Lethality test was performed to measure the LC_{50} for all *M. annua* extracts, using Colchicine and chloroform as positive control and negative control, respectively. Both the ethanolic extract and ethyl acetate extract of *M. annua* were tested for DPPH radical scavenging activity (antioxidant activity). The ethanol extract was evaluated for possible antiproliferative activities against human leukemia (K562), breast cancer (MCF-7), cervical cancer (Hela), lung cancer (A562), and fibroblast cell lines. All the three extracts from *M. annua* were also evaluated for possible antiangiogenic activity using the *ex vivo* Rat Aortic assay. **Results:** The ethanolic extract has shown mild cytotoxic activity, especially on the MCF-7 breast cell lines as compared to ethyl acetate and hexane extract. In addition, the ethanolic extract resulted in the highest radical scavenging activity (61.3% inhibition), while all extracts have shown negligible antiangiogenic activities. **Conclusion:** The study shows that the ethanolic extract of *Mercurialis annua* from Jordan could serve as a possible source for developing anticancer and antioxidant drugs in the future.

Keywords: *Mercurialis*, Antiangiogenic, Quercetin, Antioxidant, Cytotoxic.

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INTRODUCTION

Medicinal plants have been always important to mankind, animals, and environment as they are considered valuable sources of bioactive compounds. The primary and secondary metabolites in medicinal plants exert a wide range of biological activities. Many of which have been studied by humans for their beneficial role in diverse medical applications, especially for their antimicrobial and anticancer activity.[1-2] The most important of the bioactive constituents produced by plants are the secondary

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metabolites which are considered the key drivers for plant pharmacological effects.[3-5] For almost all countries worldwide, medicinal plants becoming popular solutions for many diseases and ailments owing to their lesser side effects and the potential activity they possess.[6] Medicinal Plants are the richest bio-source of drugs that have been used in traditional medicine to prepare the large number of nutraceuticals, pharmaceuticals, and intermediates to other chemical entities like synthetic drugs. According to estimates, about 70-80% of developing countries rely on medicinal plants for treatment.[7] The search for plants as a source for potential drug candidates is still unsound. It is estimated that, out of 350,000 to 500,000 species available on earth only about 10% are being potentially studied and used for their medicinal applications.[8] Cancer in particular, has captured great research interest as a result of its high mortality and the absence of selective anticancer agents in the market.[9] Various medicinal plants have been used in daily life to treat not only cancer but also microbial infections, diabetes, and cardiovascular diseases.[10-11] In developing countries, about two-third of the population relies on the use of medicinal plants for the treatment of many health problems including cancer. It has been reported that the primary healthcare system in rural areas mainly depended on traditional medicine, where 80% of the people use medicinal plants.[12-13] In Jordan, the herbalists and traditional health practitioners have used more than 145 medicinal plant herbs in traditional herbal medicine.[14] Many of these herbs are used in folk medicine for the treatment of cancer.[15] Medicinal plants are emerging as an alternative and safe choice for therapy, having the probability to reduce the drug resistance and undesirable toxicity that are always accompanied by chemotherapy treatment.

Among the most promising plants in this circumstance is *Mercurialis annua* from the Euphorbiaceae family which comprises 300 genera and 7500 species.[16] *Mercurialis annua* has been traditionally used by native people for its diuretic, laxative, antiemetic and antioxidant activity in addition to its usefulness in the treatment of warts, eye problems and microbial infections.[17-18] In addition, the methanolic extract from the ariel part of *Mercurialis annua* has shown promising anti-cancer, anti-inflammatory, and antimicrobial potentials.[19]

Moreover, the aqueous extract of *Mercurialis* ariel part has shown promising hypoglycemic and anticancer activity.[17,20] Previous phytochemical analysis for the major secondary metabolites of the aerial parts of this plant revealed the presence of narcissin (isorhamnetin 3-rutinoside) and rutin as the major flavanol glycosides in addition to three other minor flavanol glycosides; quercetin 3-rutinoside, isorhamnetin-3-*O*-rutinoside-4`-*O*-glucoside and isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside.[21]

Individually, the flavonoids have shown powerful antioxidant and free radical scavenging activity.[22] Rutin, in particular, can strengthen blood vessels, so it is used for hemorrhoids, varicose veins, internal bleeding, to prevent mucositis; a side effect of cancer treatment, also it is useful for osteoarthritis.[23] Quercetin, on the other hand, has shown to be helpful to prevent the damage related to free radicals and thereby reducing the probability of the development of a variety of conditions including inflammation, arthritis, allergies, macular degeneration, heart disease, gout, and various forms of cancer.[24-25] Kaempferol another flavanol glycoside isolated from *M. annua*, helps to prevent oxidative damage to human body cells, also prevent atherosclerosis and because of its antioxidant potential, it has been useful in cancer chemoprevention.[26-27] Another important flavanol is Isorhamnetin, which has been used as an antioxidant and antiviral effect.[28]

In the present study, different extracts of the aerial part of *M. annua* collected from Jordan territories have been tested and evaluated for possible antioxidant, cytotoxicity, and anti-angiogenic activity.

Up to our knowledge, this is the first time that extracts of *M. annua* from Jordan have been studied for potential antioxidant, cytotoxicity, and anti-angiogenic activity.

MATERIALS AND METHODS

The plant was collected from different localities in Jordan by Professor Daoud Al-Eisawi, Plant taxonomist, Department of Biological Sciences University of Jordan. A herbarium specimen (Pharm-2017-03) was deposited at the Department of Pharmacy, Faculty of Pharmacy and Medical Sciences, Amman Arab University, Amman, Jordan. Melting points were determined on a Stuart Scientific melting point apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Avance Ultrashield spectrometer (Bruker, Ettlingen, Germany) in $CDCl_3$ as part per million (d) using trimethylsilane as an internal standard. Mass spectra were measured in a positive ion mode using the electrospray ion trap (ESI) technique on a Bruker Apex-4 instrument (Germany). Thin-layer chromatography was made on silica gel (pre-coated -GF 254 Merck). Standard flavonoids were purchased from SIGMA- Aldrich, USA. Growth factors Matrigel™ was purchased from Corning, USA. Fetal bovine serum (FBS) was purchased from Gibco, Invitrogen, USA.

Extraction and fractionation

One kilogram of the dried powdered aerial parts of *M. annua*, was extracted by maceration with:

1. 4L *n*-hexane three times for 24 hr.
2. 4L Ethyl acetate three times for 24 hr.
3. 4L Ethanol 96% three times for 24 hr.

Preliminary analytical TLC was made for the ethyl acetate and ethanol extracts using solvent system toluene: ethyl acetate (9.3:0.7). Each extract was concentrated by rotatory evaporator at 40°C and kept aside in the refrigerator (at 4°C) for further analysis.

Extraction and identification of flavonoids

Two detection methods were applied for the identification of flavonoids; the first by the use of UV spectrophotometer at a wavelength 254 nm then spraying TLC plates by fast blue spray reagent followed by ammonia and comparison of the R_f value with the standard flavonoid compounds. The other method used was the spectrophotometric identification (NMR and MS) in addition to the measurement of melting point in comparison with the standard flavonoid previously purchased for this purpose.

50 gm of the concentrated ethanol extract was used for the separation of flavonoids, The extract was subjected to column chromatography (dry packing method) using silica gel (70-230 mesh, Merck) and eluted with the solvent system ethyl acetate: ethanol: water 66:15:20. Four fractions were obtained named F1, F2, F3, and F4. Each fraction was TLC Plated using the same eluting solvent system along with the standard flavonoid compounds for R_f value comparison. All four fractions were then subjected to preparative TLC using silica gel GF 254 (1mm thickness), after which the compounds were separated by scratching of the compound's TLC band that was rinsed with ethyl acetate then filtered. Filtrates were evaporated to dryness to be identified by spectrophotometric methods.

Brine shrimp cytotoxicity (BSLT)

The brine-Shrimp cytotoxicity/Lethality test was performed against *Artemia salina* (brine-shrimp eggs) in accordance with the procedure described by Meyer and his co-workers[29] by slightly modified method.[30]

38 g of sea salt was dissolved in 1L distilled water. On the top of small spatula, brine-shrimp eggs were taken and placed in 250 mL plastic container, waiting until the eggs were hatched under continuous illumination at 30°C for 48 hr and keeping the solution in the fume hood.

20 mg from each plant extract; hexane, ethyl acetate and ethanol, was dissolved in 2 mL chloroform to form the stock solution (1000 ppm) prior to dilution steps. Different dilutions have been prepared by taking 0.2 mL from stock solution and dilute it with 1.8 mL of ocean water to form, 1000, 100, 10, and 1 ppm to be tested. 0.5 mL from each concentration was placed in 7 mL glass vials, and left to evaporate under fume hood for 12 hr overnight. 10 brine shrimp were loaded to each vial and ocean water was added to get 5mL final volume. All vials (in triplicate) were incubated at 30°C for 24 hr. After this, the vials were visually inspected and counting of the number of survived nauplii in each vial was carried out by using magnifying glass.

In vitro antioxidant evaluation

The 2,2-diphenyl-l, picryl hydrazyl (DPPH) method described by Kulisic[31] and slightly modified by Obeid group[32] was applied to evaluate the antioxidant potential for both ethanol and ethyl acetate extracts of *M. annua*. The hydrogen atom donating ability of corresponding extracts were measured from the bleaching of purple methanolic DPPH solution. Aliquots (2.5-800 µg/mL) of the tested samples were mixed with 950 µg of 0.1mM DPPH in absolute methanol. Reduction of DPPH radical was determined by measuring absorbance at 517 nm after 30 min against a blank without DPPH. The results were expressed as the percent inhibition of DPPH radical and calculated as $[(A_{0-} As)/A0 \times 100]$, where A_0 = Absorbance of DPPH without sample and As is the absorbance of DPPH with sample. Ascorbic acid was used as the positive control.

Anti-proliferative activities

The effect of *M. annua* extracts on Human Leukemia (K562), breast cancer (MCF-7), cervical cancer (Hela), lung cancer (A562) and Fibroblast cell lines were evaluated. Media were supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA), 1% of 2 mM L-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin (Lonza, Belgium). Cells were seeded at a density of 7×10^3 (8×10^3 for fibroblasts) cells/well in 96-well plates and allowed to attach overnight. The cells were treated with two different concentrations (20 and 100 µM) of extracts in triplicates. After 72 hr treatment, MTT assay was performed according to cell proliferation assay kit (Promega, USA). DMSO was used as a negative control and percentage viability inhibition was measured. Absorbance (OD) was measured at 570 nm. All cells were maintained at 37°C, with 5% CO_2 in a humidified incubator.[33]

Anti-angiogenic testing (*ex vivo* Rat Aortic assay)

The Rat Aortic ring assay was performed according to the legally approved document by the Animal Ethic

Committee of the University of Jordan. 200-250g Spargue Dawley male rats were used for this test. The specified animal specimens were brought from the animal house facility of the University of Jordan. After the anesthetization of rats, the thoracic aorta was excised and immediately placed in cold phosphate buffered saline (PBS) solution. By using a dissecting microscope, the fibro-adipose tissue was removed carefully, and aorta cleaned, then cross sectioned into 1mm thick rings. The sectioned rings transferred into a fresh cold PBS solution. The rings were placed in a pre-cold 48 well plate, one ring for each well, and covered by a layer of 30 µL low growth factors Matrigel™ (Corning, USA) that was kept on ice during the whole procedure. The rings were seeded in triplicates. After that, the plate incubated at 37°C for 30 min to ensure Matrigel solidification. A 250 µL of RPMI media containing plant extracts at a concentration of 100 µg/mL was added to each well. DMSO was used as a negative control at 1% (v/v). On day four, the media was removed and replaced with new fresh amount of *Mercurialis* extracts with the same concentrations. On day six, and by use of inverted light microscope at 4x magnification, the rings were photographed. For analysis, ImageJ software (National Institute of Health, Bethesde, MD) is usually used to measure the magnitude of blood vessel out-growth. Furthermore, the blood vessels formation percentage of inhibition is calculated using the following Formula:

$$\% \text{ Blood vessels inhibition} = (1-(A_0/A)) *100$$

Where, "A_0" is the distance of blood vessels growth in treated rings in arbitrary units and "A" is the distance of blood vessels growth in the negative control in arbitrary units.[34]

RESULTS AND DISCUSSION

Four flavonoids have been isolated from the ethanol extract; namely Kaempferol, Isorhamnetin, Quercetin, and Rutin. The R_f values for the isolated flavonoids were compared to reference compounds using the thin layer chromatography (TLC) and ethyl acetate/ethanol/water (65/10/25) as the mobile phase. The R_f values obtained were 0.99 for Kaempferol, 0.95 for Isorhamnetin, 0.87 for Quercetin, and 0.15 for Rutin. Also, the melting points for the isolated flavonoids were measured to be 277°C for Kaempferol, 307°C for Isorhamnetin, 314°C for Quercetin, and 242°C for Rutin which are comparable with the reported melting points in literature.[22] All isolated flavonoids were identified further by ^{1}H NMR and MS for characterization (Table 1).

Brine shrimp lethality test (BSLT)

The brine shrimp bioassay was performed to measure the LC_{50} for all *M. annua* extracts. The percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. Colchicine (reference standard) and chloroform were used as positive control and negative control respectively. All the procedures were replicated. The collected data were analyzed using Finney computer program (Probit analysis, software STATISTICA.) to determine LC_{50} values.

The numbers of survivors were counted and percentages of deaths were calculated. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The LC_{50} in µg/mL was calculated from the regression equations obtained from the graphs using the statistical method. Abbot's formula was used to correct the values, i.e., P = Pi-C/1-Cn where P denotes the observed non-zero mortality rate and C represents the mortality rate of the control group. The extract with LC_{50} below 1.0 µg/mL is considered highly toxic, whereas extracts with LC_{50} between 1.0-10 µg/mL is considered toxic. The LC_{50} for *M. annua* extracts are shown in Table 2.

According to above results, the 3 extracts of *M. annua* (hexane, ethyl acetate and ethanol have shown weak cytotoxic activity with mild difference in activity between them. The most toxic one to the brine shrimp was the ethanol extract that need further investigation to evaluate their cytotoxic activity especially on cancer cell lines.

DPPH Radical Scavenging Activity

Both the ethanolic extract and ethyl acetate extract of *M. annua* were tested for DPPH radical scavenging activity at a concentration of 1mg crude extract/mL. Extracts showed adequate antioxidant activity. The highest (%) DPPH radical scavenging activity was shown by ethanol extract of *M. annua* (61.3%) at the concentration of 381.1µg/mL (Figure 1) comparing with Rutin which showed 84.5% at the concentration of 80.0µg/mL, while ethyl acetate extract showed 20.2% inhibition of DPPH. In addition, hexane extract was the least active with 22.5% inhibition even at higher concentration (800 µg/mL).

Antiproliferative Activity

The ethanol extract was the only extract tested for possible cytotoxicity on both normal fibroblast and cancer cell lines. At a concentration of 100 µM the extract

Table 1: H^1 NMR and MS Data for the isolated compounds.

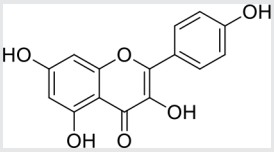
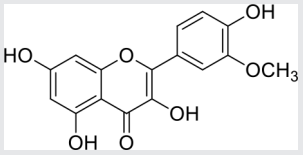
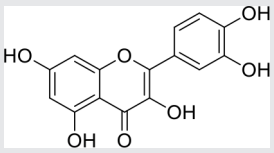
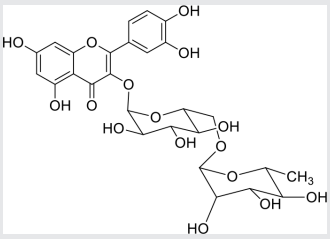
Compound's name	Chemical structure	NMR Data	MS Data
Kaempferol (m.p 277°C)		6.15 (*d*, 1 ArH, *J*= 3.4 Hz), 6.39 (*d*, 1 ArH, *J*= 3.4 Hz), 6.87 (*d*, 2 ArH, *J*= 17.6 Hz), 8.01 (*d*, 2 ArH, *J*= 17.6 Hz), 9.33 (*bs*, 1OH), 10.05 (*bs*, 1OH), 10.72 (*bs*, 1OH), 12.43 (*s*, 1OH)	593(M-H), 285 (100%), 284(44%)
Isorhamnetin (m.p. 307°C)		3.81 (s, OCH_3), 6.15 (*s*, 1 ArH), 6.43 (*s*, 1 ArH), 6.91 (*d*, 1 ArH, *J*= 16.9 Hz), 7.66 (*d*, 1 ArH, *J*= 16.9 Hz), 7.71 (*s*, 1 ArH), 9.36 (*bs*, 1OH), 9.68 (*bs*, 1OH), 10.72 (*bs*, 1OH), 12.42 (*s*, 1OH)	623 (M-H), 315, 314 (100%), 300 (100%)
Quercetin (m.p 314°C)		6.15 (*d*, 1ArH, *J*= 3.3Hz), 6.37 (*d*, 1 ArH, *J*= 3.2Hz), 6.86 (*d*, 1 ArH, *J*= 16.9 Hz), 7.51 (*dd*, 1 ArH, *J*= 16.9, 3.7 Hz), 7.64 (*d*, 1 ArH, *J*= 3.8 Hz), 9.28 (*bs*, 2OH), 9.52 (*bs*, 1OH), 10.72 (*bs*, 1OH), 12.44 (*s*, 1OH)	625 (M-H), 463, 300(100%)
Rutin (m.p 242°C)		0.96 (*s*, CH_3, *J*= 12.3 Hz), 3.03 (*m*, 2CH), 3.23 (*m*,5CH), 3.68 (*d*, CH, *J*= 21 Hz), 4.35 (*s*, CH), 5.05 (*m*, CH_2), 5.31 (*d*, CH, *J*= 14.1 Hz), 6.15 (*d*, 1 ArH, *J*= 3.5 Hz), 6.34 (*d*, 1 ArH, *J*= 3.5 Hz), 6.81 (*d*, 1 ArH, *J*= 16.2 Hz), 7.51 (*m*, 2 ArH), 9.12 (*bs*, 1OH), 9.61 (*bs*, 1OH), 10.77 (*bs*, 1OH), 12.55 (*s*, 1OH)	609 (M-H), 301 (60%), 300 (100%)

Table 2: Results of Brine Shrimp Lethality Test (BSLT).

Re analyzed Sample	The Number of Survivals After 24 hr				LC_{50} (µg/mL)
	1000 ppm (µg/mL)	100 ppm (µg/mL)	10 ppm (µg/mL)	1 ppm (µg/mL)	
Hexane extract	0	2	10	15	1.15
Ethyl acetate extract	0	8	7	14	0.95
Ethanol extract	2	5	6	14	0.85

has shown high toxicity against normal fibroblast cells. At lower concentration (20 µM), the extract exerted a very mild toxicity towards fibroblast (24.03%) but did not show promising toxicity on the different cancer cell lines (Table 3). According to these results this extract did not possess any promising antiproliferative activity, and these results are in accordance with the previously obtained results using the methanol extract on breast cancer cells (MCF-7).[20]

Ex vivo Rat Aortic Assay

Angiogenesis plays an important role in the formation of a new vascular network to supply oxygen and nutrients, while removing waste products. Thus, the angiogenic and lymphangiogenic factors are important for metastasis of cancer cells and increasingly receiving

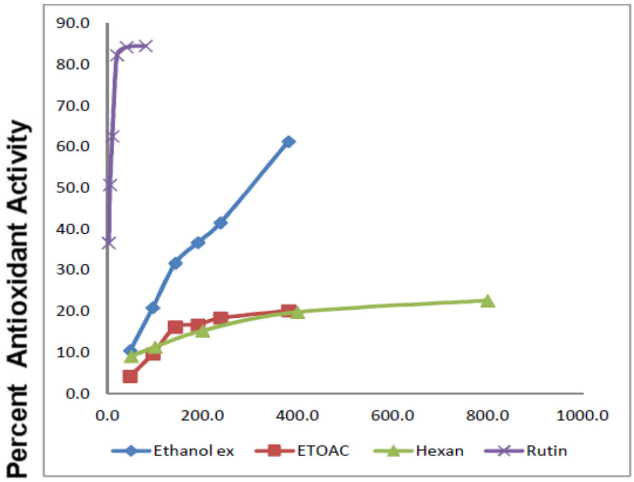


Figure 1: Antioxidant activity of ethanol, ethyl acetate and hexane extract extracts.

Table 3: Results of antiproliferative activity for ethanol extract of *M. annua.*

Cell Line	% Inhibition	
	100 µM	20 µM
MCF7	20.74 ± 6.96	15.71 ± 2.77
K562	28.52 ± 0.57	2.09 ± 1.81
Hela	14.44 ± 2.16	2.09 ± 1.33
A562	10.33 ± 2.75	6.89 ± 6.61
Fibroblast	69.03 ± 1.63	24.03 ± 1.27

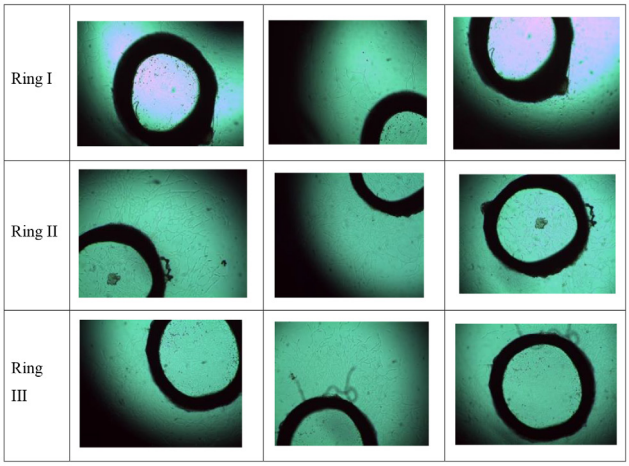


Figure 2: The *ex vivo* Rat Aortic assay of *M. annua* hexane extract.

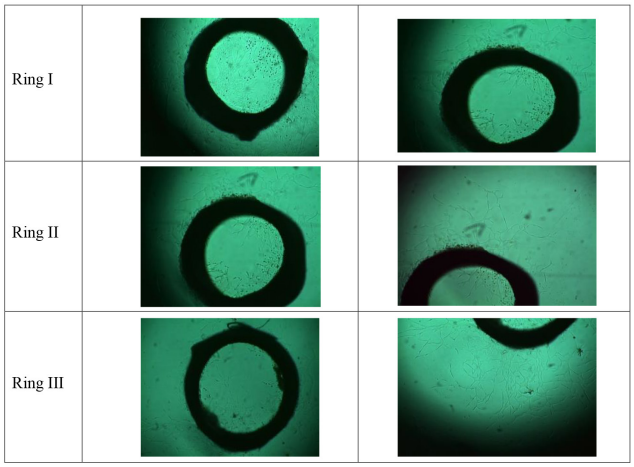


Figure 3: The *ex vivo* Rat Aortic assay of *M. annua* ethanol extract.

attention in the last 10 years, especially in the field of neoplastic vascularization.

Thus, the three extracts from *M. annua* were evaluated for possible antiangiogenic activity using the *ex vivo* Rat Aortic assay (Figures 2-4). Unfortunately, all the *Mercurialis* extracts have shown the negligible effect on the magnitude of blood vessel out-growth.

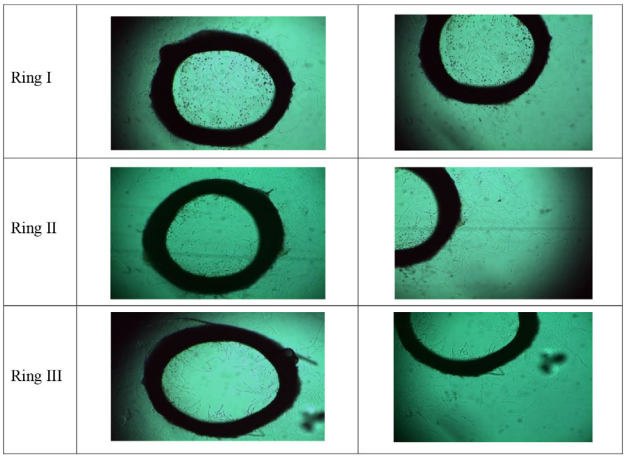


Figure 4: The *ex vivo* Rat Aortic assay of *M. annua* Ethyl acetate extract.

CONCLUSION

Mercurialis annua belonging to the family *Euphorbiaceae* is known for its cytotoxic, anti-inflammatory, and hypoglycemic activities. In this study, *M. annua* from Jordan territories has been evaluated for possible antioxidant, cytotoxic, and antiangiogenic activity. For both antioxidant and cytotoxic activity, the ethanolic extract was the most promising extract to be further studied and its constituents have to be analyzed. The ethyl acetate and hexane extract, on the other hand, did not show cytotoxic or antioxidant activity. The antiangiogenic effect was negligible for all the extracts which means that the cytotoxic effect of the ethanolic extract is not because of its effect on angiogenesis. Further studies should focus on studying the ethanolic extract in order to examine its cytotoxic mechanism of action as well as the active constituents responsible for this tangible effect.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CO_2: Carbon dioxide; **DMSO:** Dimethyl sulfoxide; **DPPH:** 2,2-diphenyl-l, picryl hydrazyl (DPPH); **LC_{50}:** Lethal Concentration 50; **MS:** Mass spectrometry; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **NMR:** Nuclear Magnetic Resonance;

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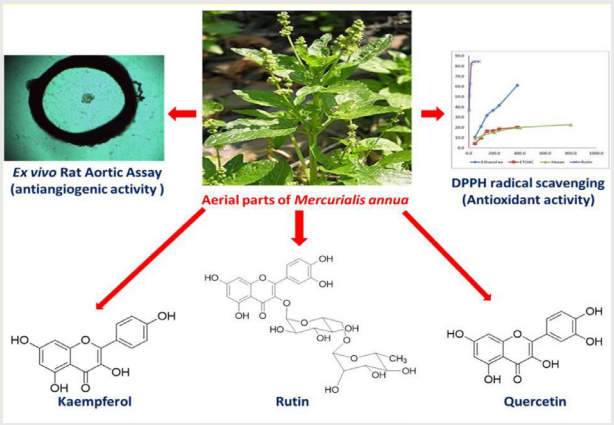
PICTORIAL ABSTRACT



SUMMARY

Aerial parts of *Mercurialis annua* were collected from the Jordan territories and extracted by maceration with n-hexane, ethyl acetate, and ethanol (96%), respectively. Extracts were tested for possible anti-oxidant, cytotoxic and antiangiogenic activities. The ethanolic extract has shown mild cytotoxic activity, especially on the MCF-7 breast cell lines as compared to ethyl acetate and hexane extract. In addition, the ethanolic extract resulted in the highest radical scavenging activity (61.3% inhibition), while all extracts have shown negligible antiangiogenic activities.

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