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Antioxidant and MDR reversal activity in resistant human ovarian cancer cells of methanolic extract from *Ruta Montana* located in the North of Algeria

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ABSTRACT

The aim of this work is to determine the in-vitro antioxidant and multi-drug resistant (MDR) reversal activity in resistant human ovarian cancer cell line (A2780 DX3) of Ruta Montana methanolic extract (RMME), a perennial aromatic herb originated from North Eastern Algeria belongs to the family Rutaceae. The preliminary phytochemical screening of the extract revealed the presence of flavonoids, tannins, saponins, coumarins and alkaloids. Quantitative determination of total phenolics and total flavonoids was carried out using colorimetric methods. The total phenolic content was found to be142.33 mg of gallic acid equivalent per gram of extract, while the content of flavonoid show a value of 23.093 mg of quercetin equivalent per gram of extract. The antioxidant activity was evaluated in vitro with the use of free radical scavenging activity method by DPPH assay, the result expressed in terms of IC50 was found to be 0.12 mg/ml. On the other hand, the synergistic property of RMME with doxorubicin was analysed on A2780 DX3 resistant cell line using MTT assay. The ability of various concentrations of RMME to reverse MDR to doxorubicin in A2780 DX3 cells was investigated by the MTT method in the presence of doxorubicin (7µM). The concentrations 10 and 40 mg/ml of RMME overcame the MDR with the reversal fold (RF) values of 2.01 and 4.56 respectively, but the concentrations 0.625 and 2.5 mg/ml were weakly active. Finally, from results it can be concluded that methanolic extract contains the most active secondary metabolites and is the potential candidate to isolate the active compounds responsible for the effects observed with R.montana.

Key words: *Ruta Montana*, Antioxidant activity, DPPH$^{\bullet}$, MDR reversal, MTT assay.

INTODUCTION

Medicinal plants have been used as remedies for human diseases for centuries. The reason for using them as medicine lies in the fact that they contain chemical components of therapeutic value. The medicinal value of plants lies in some chemical substances (usually secondary metabolites) that produce a definite physiological action on the human body [1].

Ruta species (*Rutaceae*) are sources of different classes of natural products such as flavonoids, alkaloids, essential oils, coumarins, phenols, saponins lignans, and triterpenes, with biological activities, including antifungal, antioxidant, phytotoxic, abortive depressant, antidotal and anti-inflammatory. There are four *Ruta* species and subspecies in Algeria, *R. montana*, *R. chalepensis* subsp. *angustifolia*, *R. chalepensis* subsp. *latifolia* and the Saharian species *R. tubercula*. [2, 3, 4]

Ruta montana (Clus.) in Arabic *"fidjla"*, is a perennial aromatic herb originated from North Eastern Algeria [3]. The plant has been used in Algeria as a cure for emmenagogue, antispasmodic and rubefiant.

In recent years, numerous drug resistances (MDR) in human cancer have developed. Classical MDR is attributed to the over expression of plasma membrane P-glycoprotein (P-gp) in resistant cells which is the ATP-binding cassette (ABC) superfamily of membrane transporters. Its activity results in a marked reduction in the intracellular concentration of a wide range of substrates including anticancer drugs. Efflux of these drugs out of cells by P-gp is a reason for failure of chemotherapy. Its efflux function can be circumvented by a wide range of pharmacological agents *in vitro* and *in vivo*. However, their use in alleviating MDR is limited because the concentrations required for inhibition of the pump surpass their dose-limiting toxicity. Agents derived from plant capable of circumventing MDR with minimal adverse side effects is an attractive goal [5].

On the other hand, free radicals are known to be the major cause of various chronic and degenerative diseases. An antioxidant, which can quench reactive free radicals, can prevent the oxidation of other molecules and may therefore have health-promoting effects in the prevention of degenerative diseases. There is a growing interest in natural antioxidants, present in medicinal and dietary plants that might help attenuate oxidative damage [6].
This research was, therefore, conducted to:

- Study the antioxidant activity of RMME by DPPH° scavenging activity
- Assess synergistic property of RMME with doxorubicin (DXR) in human ovarian cancer multidrug- resistant cell line (A2780 DX3) using MTT assay.

MATERIALS AND METHODS

Chemicals

The chemicals were purchased from Sigma (USA) and Fluka Chemie (Buchs, Switzerland)

2.1. Medicinal plant selected

R. montana L.- *Rutaceae*- aerial parts was used in the present investigation. Fresh *R.montana* was collected in Mai 2012 from the region of Mila (sub-humid region), Algeria. Taxonomic identification was perfomed by Pr. Z. Kabouche (Laboratory LOST, University Mentouri - Constantine, Algeria).

2.2. Preparation of *R. Montana* methanolic extract (RMME)

Fresh aerial parts (stems, leaves and flowers) were air-dried in shade at room temperature. They were then mechanically powdered and sieved. 640 g of the obtained powder were macerated during 48 h at room temperature with mixture of distilled water–methanol (3/7 V/V). The obtained crude preparation was filtered and concentrated under reduced pressure by rotary evaporator at 47 C° to give the crude methanolic extract [7].

2.3. Phytochemical Screening

The RMME was tested for the presence of flavonoids, alkaloids, tannins, saponins and coumarins. The qualitative results are expressed as (+) for the presence, (-) for the absence and (±) for traces of of phytochemicals.

2.3.1. Test for flavonoids

5 ml of RMME was added with few drops of 1% $AlCl_3$.Yellow color indicated the presence of flavonoids [8].

2.3.2. Test for alkaloids

About 50 mg of extracts was stirred with 5 ml of hydrochloric acid (2N) and filtered. Then, few drops of Wagner's reagent (2g of KI and 1,27g of I_2 were dissolved in in100 ml distilled water) were added at the side of the test tube. A brown colored precipitate indicates the presence of alkaloids [8].

2.3.3. Test for tanins

A few drops of 2% ferric chloride were added to 2 ml of RMME**.** blue- Black coloration or precipitate was taken as positive result for the presence of tannins [9].

2.3.4. Test for Saponins

5 ml of RMME was separately shaken with distilled water (10 ml) in a test tube for 2 min. The formation of frothing, which persists for 15 min, shows the presence of saponins [9].

2.3.5. Test for coumarins

In a test tube, 1 g of the extract was placed and covered with filter paper moistened with dilute sodium hydroxide (NaOH), then heated on water bath for a few minutes. The filter paper was examined under UV light, yellow fluorescence indicated the presence of coumarins [10].

2.4. Estimation of total flavonoid

Aluminum chloride colorimetric method was used for flavonoid determination in RMME.1ml of diluted extract was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 0 to 40 µg /ml in methanol. And the flavonoids content was expressed in mg per g of quercetin equivalent (QE) [11].

2.5. Estimation of total polyphenols content

Total polyphenols were measured using Prussian blue assay method described by Price & Bulter and modified by Graham [12]. Phenolic contents were expressed as gallic acid equivalents. Briefly, 0.1 ml of RMME was dissolved in methanol and 3 ml distilled water were added and mixed up. One ml of $K_3Fe(CN)_6$ (0.016 M) was added to the sample followed by the addition of 1 ml of $FeCl_3$ (0.02 M dissolved in 0.1 M HCl) and immediately mixed up using a vortex. After the addition of the reagents to the sample, 5ml stabilizer (30 ml of 1% gum Arabic, 30 ml of 85% H_3PO_4 and 90 ml distilled water) were added to the sample and mixed up. The absorbance was measured at 700 nm using a spectrophotometer. The amount of total polyphenols in the extract was determined from a standard curve of gallic acid ranging from 0.00 to 200 µg/ml.

2.6. Antioxidant studies by DPPH° Radical -Scavenging Activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH°) was used for determination of free radical-scavenging activity (RSA) of the extract. Dilutions of RMME were prepared in a concentration ranging from 0.3-0.001 mg /ml. From these dilutions 15 µl of extract was added to 1.5 ml of a 100 µM DPPH• solution in methanol. The mixtures were shaken vigorously and incubated in the dark for 15 min after which the reduction of DPPH• absorption was measured at 517 nm. At the same time, a blank solution of DPPH• with solvent used in dilutions was screened to estimate the DPPH• decomposition during the time of measurement. The experiment was repeated 3 times. Quercetin was used as standard control. The percent free radical scavenging activity was calculated as below:

Blank OD – Sample OD / Blank OD X100

The activity of extract was determined in terms of IC_{50} value denotes the concentration of a sample, which is required to scavenge 50% of DPPH• free radicals [13]. IC50 was calculated from the plotted graph of scavenging activity against the concentrations of the extract.

27. Synergistic property of RMME with DXR in human ovarian cancer multidrug- resistant cell line (A2780 DX3) using MTT assay

2.7.1. Preparation of different concentrations

Different concentrations of methanolic extract were prepared by dissolving the extract in Dimethyl sulfoxide (DMSO) (0.2%) under sterile conditions.

2.7.2. Cell lines and culture conditions

DXR resistant human ovarian carcinoma cell line (A2780 DX3) was used in that study. The cell line was obtained from laboratory of Biochemical (Sapeinza University, Rome). Cells were grown to 70-80% confluence in RPMI medium supplemented with 10% FBS, 100 g/ml streptomycin, 100units/ ml penicillin, 1mM Sodium pyruvate and 2 mM glutamine at 37°C in a humidified atmosphere of 95% air with 5% CO_2 in incubator.

2.7.3. MTT assay for multidrug resistance reversal with RMME

DXR at the concentration of 7µM with and without RMME of different concentrations were tested for reversing MDR using MTT assay. MTT is a yellow colored dye which is reduced into purple colored formazan crystals by the activity of mitochondrial succinate dehydrogenase enzyme in viable cells [14]. The cells were seeded into 96-well culture plates at 5×10^3 cells/well. Cells were treated with varying concentrations of RMME (0.625, 2.5, 10, 40 mg/ml) with 7µM DXR (8 wells per each concentration) and the medium of control culture was treated with DXR (7µM) alone in fresh media and incubated for 48 h. At the end of treatment period, 100 µL of MTT solution (5 mg/ml) was added to each well. After the addition of MTT, the plates were incubated for 3 h in dark chamber. Then the medium was discarded and100 µL of DMSO was added to well to dissolve the formazan crystals. The absorbance in individual wells was read at 570 and 690 nm using ELISA reader. The reversal fold (RF) values, as potency of reversal [15, 16] was calculated as follows:

RF= % cell viability of DXR alone / % cell viavility of DXR in the presence of various concentrations of RMME

2.8. Statistical analysis

Results were expressed as mean ± SD of three measurements.

RESULTS AND DISCUSSION

3.1. The yield of the extract

The extract was obtained after removal of methanol by evaporating under reduced pressure using rotary evaporator at 47 C°. The extraction yield of RMME is shown in Fig. (1).

The methanolic extraction yield of *R.montana* was 49%. It has been reported that the efficiency of the yield extraction of plant extract was influenced by several parameters, including chemical composition and physical characteristics of the plant material [17], the extraction time, temperature, the volume and type of the solvents used [18].

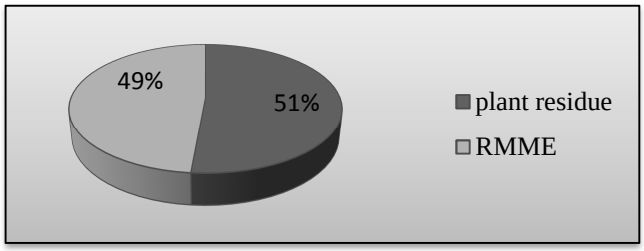

Fig. (1) : The yield of the methanolic extract from *Ruta montan*

3.2. Phytochemical screening

Investigations on phytochemical screening of crude methanolic extract of aerial part of *R. montana* revealed the presence of some secondary metabolites such as flavonoids, tannins, saponins, coumarins and alkaloids as shown in Table 1.

Table (1): Phytochemical constituents of RMME

Phytochemicals	*Ruta Montana* methanolic extract
Flavonoid test	+
Tannin test	+
Saponins test	±
Alkaloid test	+
coumarins	+

(+); presence and (±); traces of chemical constituents

All secondary metabolite identified in RMME are known to be biologically active compounds and they are responsible for different biological activities. Several reports are available on pharmacological and biochemical actions of flavonoid groups, such as antioxidant, anti-allergic, anti-inflammatory, antimicrobial and anticancer properties [19]. Flavonoids are also known to have a wide array of therapeutic activities as antibacterial [20], antiviral and anticancer activity and can be used in cancer prevention [21], They considered to be primary antioxidants or free radical scavengers [22]. Saponins are a bioactive constituent which involved in plant defense system because of their antimicrobial activity [23] and they have been reported to possess antioxidant, anti-inflammatory and hemolytic activity.

Alkaloids have been reported as powerful poison and many alkaloids derived from medicinal plants show biological activities like, anti-inflammatory [24] antimalarial [25] , antimicrobial [26] , cytotoxicity, antispasmodic and analgesic activities [27] . Coumarins are a secondary metabolites in many higher plants species especially exists in the Rutaceae and Umbrelliferae plants [28], coumarin are considered to have a wide range of biological activity, such as anti-inflammatory [29], anticancer [30, 31], anti-coagulant, anti-oxidant, as well as anti- HIV and anti-bacterial [32]. Biological activity of coumarins has becoming an appealing point of studies owing to its different effects to diseases and less damage to normal cells [33].

The corresponding results accorded with the previous finding [3] witch confirmed that *Ruta* species are sources of diverse classes of natural products such as flavonoids, alkaloids, essential oils, coumarins, phenols, saponins lignans, and triterpenes, with biological activities including antifungal, antioxidant, phytotoxic, abortive, depressant, antidotal and anti-inflammatory. The presence of above phytochemicals compounds in the *R.montana* may be the reason for its use in a traditional medicine; such as digestive disorders and helminthiasis, abortive and anti-fever effects [3]. Therefore, the detected of these bioactive compounds in RMME may be responsible for the antioxidant and MDR reversal activity.

3.3. Flavonoid and total phenol contents of the RMME

The total phenolic content of RMME was estimated by the prussian blue assay method, the result in terms of gallic acid equivalent per gram of extract was 142.33 mg (Table 2). When total phenolic content of *R. Montana* is compared with the data available for the same genus and for the same family, it is found that it has a higher value. *Ruta Montana* showed significantly higher concentrations of total phenolics compared to the total phenolic content of methanolic extract of *R. tuberculata* (*20.36±0.4* mg GAE/ g dry weight) growing in East Algerian Sahara.

Table (2) also show the content of flavonoids that was estimated by using aluminium chloride colorimetric technique in terms of quercetin equivalents as 23.093mg of quercetin equivalent per g of extract. These values are comparable to flavonoid content of the same genus. For example flavonoid contents of *Ruta tuberculata* growing in Algeria is 19.67 ±0.34 mg QE/ g dry weight.

Therefore, it can be said that polyphenolic, and flavonoid may work together with other phytochemicals present in *R. Montana* and make it medicinally important because they help human body to fight against diseases. It has been recognized that flavonoids and polyphenolic show biological activity and their effects on human nutrition and health are considerable.

Table (2): Total polyphenol and flavonoid contents of RMME extract

	Total polyphenol and Flavonoids (mg)	
RMME	**mg equivalent Gallic acid / g dry weight**	**mg equivalent quercetin / g dry weight**
	142.33± 2.33	23.093±0.95

Data were expressed as means ±SD.

3.4. Antioxidant studies by DPPH° radical -scavenging activity

The antioxidant ability and radical scavenging activity (RSA) of plants are associated with its medicinal values [34]. The antioxidant activity of the RMME and standard quercetin at different concentrations are studied by DPPH• method which remains one of the most used methods for assessing the radical scavenging activity [35]. The role of DPPH• method is that the antioxidants react with the stable free radical. During the free radical reaction, DPPH• (a,a-diphenyl-b-picrylhydrazyl) is converted into DPPH-H (a,a-diphenyl-b-picrylhydrazine) with color change from purple to yellow, The rate of color change gradually decreases to indicate the scavenging potentials of the sample antioxidant [36]**.** The results are given in Table (3) and Fig. (2).

Table (3). Radical scavenging activity of RMME and quercetin

Concentration of RMME (mg/ml)	% of RSA of RMME	% RSA of quercetin
0.3	91.64 ± 0.28	96.10 ± 0.19
0.2	74.36 ± 0.26	95.90 ± 0.23
0.1	52.23 ± 0.063	95.82 ± 0.05
0.05	39.69 ± 0.13	95.88 ± 0.16
0.03	32.1 ± 0.07	95.75 ± 0.05
0.02	30.94 ± 1.52	95.94 ± 0.16
0.01	28.33 ± 1.77	95.94 ± 0.06
0.005	18.63	95.97 ± 0.19
0.0025	17.24 ± 0.60	95.01 ± 0.35
0.00125	13.91 ± 1.88	46.45 ± 1.78
0.001	12.76 ± 1.31	42.52 ± 1.64

Data were expressed as means ±SD.

The antioxidant activity of the extract and standard increased as the concentration increased but the RMME exhibited low radical scavenging activity than the quercetin across all concentrations examined, where the results of RMME show maximum inhibition of DPPH 91.64 % at the concentration of 0.3mg/ml, while at the same concentration quercetin show 96.10 %.

The extract and quercetin show IC50 values of 0.12 mg/ml and 0.0013 mg/ml respectively Fig. (2).

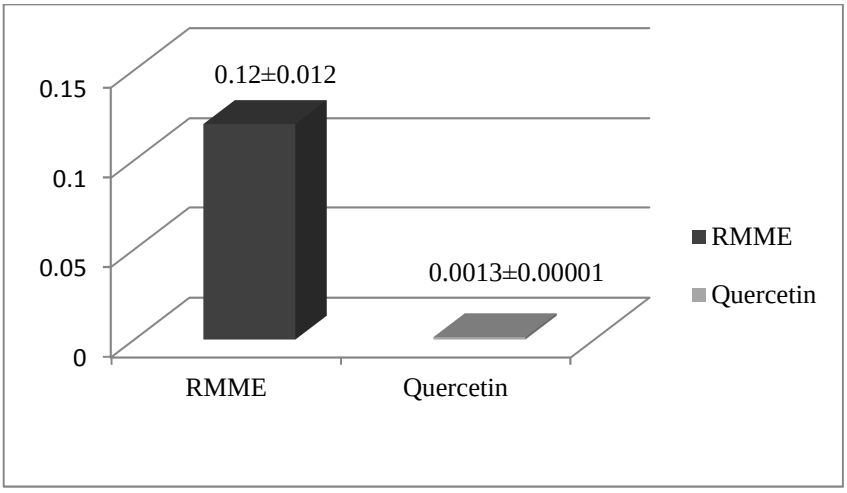

Fig. (2): IC_{50} (mg/ml) values of RMME and quercetin for RSA by DPPH˙

Since the lower IC_{50} value means higher antioxidant activity, therefore RMME show potential antioxidant activities because when comparing this IC_{50} value with other medicinal plants, it was observed that *R.montana* has high values, once again confirming its greater potential antioxidant activity with other medicinal plants [37, 38].
The RMME contain flavonoid, saponins, tannins and phenolic compound. All these bioactive compounds were able to discolor DPPH˙ solution by their hydrogen donating ability [23].

3.5. Synergistic property of RMME with DXR in A2780 DX3 cell line using MTT assay.

The ability of various concentrations of RMME to reverse MDR to DXR in A2780 DX3 cells was investigated by the MTT method in the presence of a non cytotoxic concentration of doxorubicin (7µM). Percentage of cell viability values for DXR in the presence of different concentration of RMME was calculated and the reversal folds (RF) were evaluated.

Table (4). Effect of various concentrations of RMME on DXR cytotoxicity in A2780 DX3 cells

compounds	% cell viability	Reversal fold (RF)
Doxorubicin7µM	30.01 ± 4.55	-
Doxorubicin7µM + RMME (0.625 mg/ml)	22.28 ± 8.78	1.34
Doxorubicin 7µM + RMME (2.5 mg/ml)	21.09 ± 4.04	1.37
Doxorubicin 7µM + RMME (10 mg/ml)	14.94 ± 6.14	2.01
Doxorubicin 7µM + RMME (40 mg/ml)	6.57 ± 2.18	4.56

Data were expressed as means ±SD.

The results obtained in Table (4) showed that at the concentrations 10 and 40 mg/ml, RMME overcame the multidrug-resistance (MDR) with the reversal fold (RF) values 2.01 and 4.56 for A2780 DX3 cells, respectively, but the concentrations 0.625 and 2.5 mg/ml were weakly active. In summary, the RMME differentially increased the toxicity with DXR in the A2780 DX3 cells.

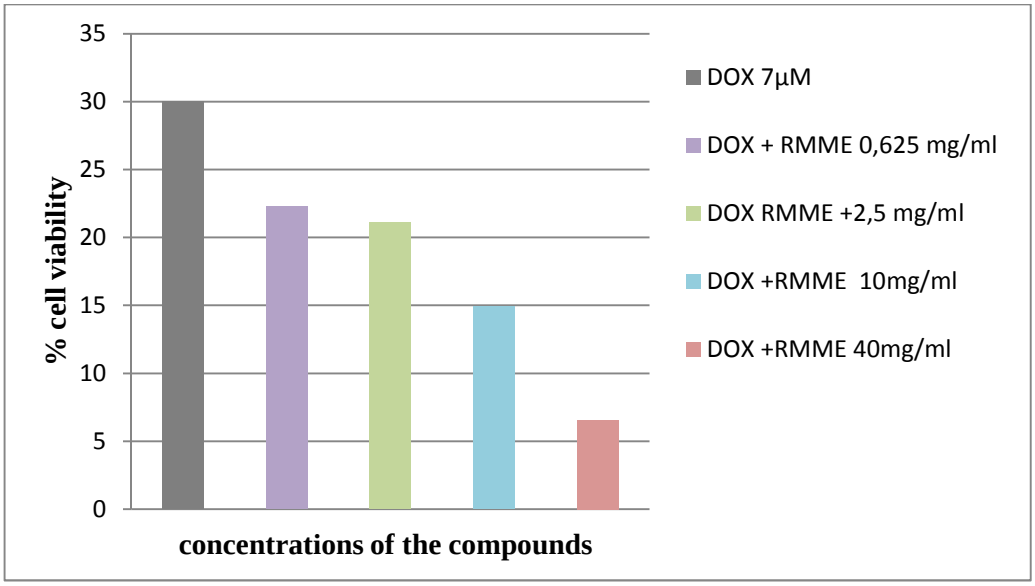

Fig. (3): MTT assay for multidrug resistance reversal with Different concentrations of RMME

The percentage of cell viability in the presence of DXR of A2780 DX3 cells was decreased as the concentration of the extract increased. These results suggest that RMME increased the toxicity with DXR in the A2780 DX3 cells.
It is well known that MDR is a major cause of failure of cancer chemotherapy. The main characteristic of MDR tumor cells is the overexpression of membrane transport proteins of ATP-binding cassette (ABC) family [39, 40], which actively transport drugs out of cells, resulting in a decrease of intracellular drug concentration. The first identified ABC transporter is P-glycoprotein (P-gp, also named as MDR1) [41].

Many attempts have been proposed to overcome MDR, One of the effective ways to overcome P-gp-mediated drug resistance is either to block its drug pump function or inhibiting MDR1 gene expression. A number of agents, known as inhibitors, modulators, or chemosensitizers like Calcium channel blockers such as verapamil have been attempted to reverse P-gp-mediated MDR by blocking drug-pumping function of P-gp. However, these agents exhibit dose-limiting side effects that severely restrict their clinical utility [42].

Therefore, much effort is currently being expanded toward identifying natural compounds from plant origins; capable of circumventing MDR with minimal adverse side effects [43]. Phytotherapy which employs extracts and not single chemical entities is still used in many countries to treat various human diseases and health disorders. Medicinal plants produce complex mixtures of secondary metabolites consisting of alkaloids, polyphenols, or diverse saponins. As a consequence extracts contain mixtures of several main and hundreds of minor components [44] each constituent in the mixture may act independently, resulting in additive effects. However, there is good evidence that some combinations of individual secondary metabolites in an extract may exert synergistic effects. Therefore, synergy appears to be a main principle in phytotherapy [45]. Several herbal constituents, often used by cancer patients, are employed as complementary and alternative medicines (CAMs); they can overcome MDR of cancer cells by interacting with many molecular and cellular targets. Moreover, natural products can modulate or inhibit ABC-transporter activity and/or expression [46].

In this study phytochemical screening and quantitative analysis of RMME revealed that this extract contains polyphenols, flavonoids, tannins, saponins, coumarins and alkaloids. Recently, the effects of many natural occurring polyphenols, terpenoids, and alkaloids on ABC-transporters were reviewed [47].

Among polyphenols, Flavonoids have been extensively studied as chemosensitizers in a number of cancer cell lines. They have been attributed with the ability to suppress drug resistance in some cell lines by inhibition of P-gp overexpression on the surface of these cells [48] or by inhibiting Pgp-mediated cellular efflux as a result of direct interaction between the flavonoids and the protein [49] However several flavonoids have been reported to inhibit the breast cancer resistance protein (BCRP) encoded by the ABCG2 gene. thus flavonoids may represent an alternative treatment for patients who have become resistant to conventional therapy. In cancer patients receiving chemotherapy an increased intake of flavonoids could lead to adverse effects [50].

Previous study had demonstrated that an alkaloid extract from *Chelidonium majus*, has the ability to overcome MDR of different cancer cell lines through interaction with ABC-transporters [48].

Another study indicated that Stemona alkaloids may play an important role as a P-gp modulator as used *in vitro* and may be effective in the treatment of MDR cancers [51]. It has been demonstrated that phytochemicals, including alkaloids, phenolics, and terpenoids, alone or in combination with the saponin reverse the relative MDR of Caco-2 and CEM/ADR5000 cells to the chemotherapeutical agent DXR [52]. Finally, to unequivocally identify the mechanism of *R.Montana* MDR reversal activity would first require the isolation and identification of pure active compounds from *R.Montana* extract.

CONCLUSION

In conclusion, the *R.montana* methanolic extract was confirmed to exhibit antioxidant and can reverse DXR resistance in human A2780 DX3 cells, by possible inhibition of the function of P-gp or inhibiting MDR1 gene expression. The free radical scavenging assay suggest that probably phenolic compound in the extract has an antioxidant activity. In addition, The ability of RMME to reverse MDR to DXR in A2780 DX3 cells by MTT assay suggest that *R.montana* also contains a compound which exhibited a MDR reversing activity *in vitro*. Finally, to unequivocally identify the mechanism of antioxidant and MDR reversal activity in A2780 DX3 cells would first require the isolation and identification of molecular structures of pure active compounds from RMME.

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