Therapeutically Potential of *Medicago sativa* Extracts

Chemical and *in vitro* assessments

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The present study was aimed to evaluate total phenols, flavonoid and flavonols content and to assess relative cytotoxicity of Medicago sativa hydro-alcoholic and alcoholic leaves and stems extracts on human lung carcinoma (A549) and human breast carcinoma (MDA-MB-231). All extracts tested have proven to be rich in hydroxylated compounds, larger amounts of phenolic compounds were found in extracts obtained using 70% ethanol, a proper polar solvent for this molecule types. Evaluation of antioxidant activity reveals values all most comparable with the ones of ascorbic acid. The extracts induced a cytotoxic effect on both tumor cell lines in a concentration-depend manner.

Keywords: alfalfa, phenols, flavonoids, antioxidant activity, cytotoxicity

Medicinal plants prove to be a challenge for researchers from different areas, such as: food, cosmetics, pharmaceuticals, medical, due to their biological properties and lack of adverse effects compared to synthesis compounds. Considering the increased interest for human health, and being explored for the treatment and prophylaxis of many diseases, the plant material should be carefully selected from verified and certified sources because, in addition to biologically active principles, it can also contain toxic compounds (*e.g.* heavy metals) [1].

Medicago sativa L. a member of the Leguminosae family, is the main representative of the *Medicago* species from over 50 types and is known under various names like *father of all foods, the king of forage, feed queen*, alfalfa, lucerne etc. [2,3]. In the present is the most cultivated fodder worldwide but, is well-known since antiquity for treatment of different illness related to kidneys, digestive tract, asthma, inflammatory and others [4,5]. Recent studies, based on modern technology, have highlighted multiple biological properties that it possesses: estrogenic and antidiabetic activities, lowering cholesterol and triglyceride blood levels, antioxidant, antimicrobial, anti-inflammatory, anti-angiogenic, anticancer, cardioprotective, antianxiety [3,4,6-8].

Regarding the chemical composition, lucerne contains multiple classes of nutrients being rich in essential amino acids (valine, leucine, threonine and lysine), chlorophyll and vitamins (C, E, B1, B2, B6, B12, niacin, folic acid, biotin, inositol, choline, and β-carotene), minerals (Ca, Cu, Fe, Mg, Mn, P, Zn, Si), [7] and, also biologically active compounds like: saponins, flavonoids, carotenoids, volatiles compounds, anthocyanin, alkaloids, tannins [9]. Newly, EFSA (European Food Safety Authority) asserted *Medicago sativa* leaf extract as a safe dietary supplement wealthy in proteins and vitamins [Gatouillat 2014, Rafinska 2016]. To the best of our knowledge, the data concerning the antiproliferative / antitumor activity of *Medicago sativa* extracts are rather poor, this direction being one of real interest.

The present study was aimed to evaluate *Medicago sativa* extracts in terms of: i) total content of phenols, flavonoids and flavonols; ii) antioxidant activity (AAO) and iii) the cytotoxic potential on two tumor cell lines, namely A549 - human lung carcinoma and MDA-MB-231 - human breast carcinoma (fig. 1).

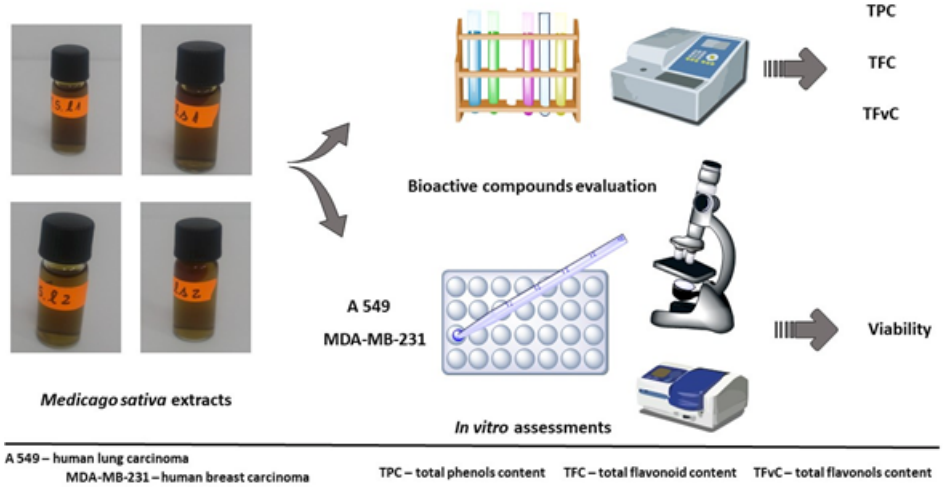

Fig. 1. Schematic protocol of the experiments conducted using *Medicago sativa* extracts

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Experimental part

Materials and methods

The reagents used: sodium carbonate (Merck, Germany), gallic acid (Sigma, Germany), ascorbic acid (Sigma-Aldrich, Germany), Folin & Ciocalteu's phenol reagent (Sigma-Aldrich, Germany), aluminum chloride (Aldrich, Germany), sodium acetate (Aldrich, Germany), rutin (Sigma, Germany), 2,2-Diphenyl-1-picrylhydrazyl (DPPH, Aldrich, Germany) and solvents were of analytical grade and were used as acquired.

Extracts preparation

Leaves and stems of *Medicago sativa* from Hunedoara county, region in Central-Western Romania, were harvested after assertive identification at the Department of Pharmaceutical Botany and a voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Victor Babes University of Medicine and Pharmacy of Timisoara. The aerial parts used in the present study were dried and subsequently subjected to ethanolic extraction. Alfalfa extracts were obtained by maceration - the plant material (1g) was left to soak for 7 days at room temperature, in 70% ethanol solution (25 mL), away from light and moisture (M.S.l1 and M.S.ls1) and by extraction at room temperature - the plant material (1g) was subjected to agitation in 96% ethanol (25 mL) by using the Orbital Shaker Incubator ES-20/60 for 24 h (M.S.l2 and M.S.ls2). After the extraction period, all samples were vacuum filtered, centrifuged, concentrated on rotary evaporator, subsequently lyophilized and stored in the refrigerator until further testing.

Phenolic content determination

Total phenolic content was estimated by using Folin–Ciocalteu test [10]. Briefly, 200 µL of each extract was treated with Folin–Ciocalteu aqueous solution in deionized water (50 µL/mL). After an incubation period of 300 s, 500 µL of sodium carbonate solution (20%) was added and the mixture was left for 2 h, at room temperature, in the dark before reading the absorbance at 765 nm using an UviLine 9400 Spectrophotometer. The calibration curve for gallic acid was obtained using ten standard solutions ranging from 50-550 µg/mL. The total content of phenols in the extracts was calculated from the calibration curve (absorbance at 765 nm *vs.* gallic acid) using the following equation determined by linear regression:

$$A = 0.0012335xC - 0.0505227 \ (R^2=0.9981552)$$

Total phenols content was expressed as mg of gallic acid / g of dry material (mg GAE / g dm). All samples were analyzed in triplicate.

Total flavonoids and flavonols content

Entire flavonoid/flavonols content was assessed by using the aluminum colorimetric assay according to Rouphael et al. method, slightly modified [11]. For flavonoid evaluation, samples (0.5 mL) of each extract were treated with 0.5 mL of aluminum chloride ethanolic solution (2%), allowed to stand for 30 min at room temperature and for flavonols evaluation samples, a volume (0.5 mL) of each extract was mixed with aluminum chloride 2% and sodium acetate 5%, and allowed to stand for three hours at room temperature. Absorbance values were read at 417 nm for flavonoid content and at 445 nm for flavonols content by using the UviLine 9400 Spectrophotometer. Rutin was used as reference standard (for calibration curve linearity range = 0–500 µg/mL, $R^2 > 0.998$) and results are expressed as rutin equivalents (RE).

Antioxidant activity evaluation

The antioxidant activity of the extracts was assessed by DPPH radical scavenging assay. Standard reagents were the DPPH solution (1 mmol/L) and ascorbic acid alcoholic solution (from Lach-Ner; 0.167 mmol/L). From each of the four alfalfa extracts, samples were prepared as follows: i) crude extract, ii) 1:5 aqueous dilution and iii) 1:10 aqueous dilution. A mixture of: 0.5 mL of the sample solution, 2 mL of ethanol 96% and 0.5 mL of 1 mM DPPH were spectrophotometrically analyzed at 516 nm on the UviLine 9400 Spectrophotometer for 20 min with a 5 s reading interval. Antioxidant activity (AAO) was calculated using the following formula:

$$AAO\ \% = 100 - \frac{A_{516(sample)}}{A_{516(DPPH)}} \cdot 100$$

where: AAO = antioxidant activity (%); A_{516} (sample) = sample absorbance measured at 516 nm wavelength at t time; A_{516} (DPPH) = the absorbance of the DPPH alcoholic solution (without sample), measured at 516 nm wavelength at t time;

The data attained were statistically analyzed with the Origin 8 software package.

Cell lines

The cell lines used in the experiments were: A549 (ATCC® CCL-185™) (human lung carcinoma) and MDA-MB-231 (ATCC® HTB-26™) (human breast carcinoma). Tumor cell lines were purchased from the American Type Culture Collection (ATCC) as frozen samples. Specific reagents required for cell cultivation, such as: Dulbecco's modified Eagle Medium - DMEM, fetal bovine serum - FBS, the penicillin / streptomycin antibiotic mixture, phosphate saline buffer - PBS, Trypsin/EDTA solution and Trypan Blue were purchased from Sigma Aldrich (Germany).

Cell culture and viability assay

Both type of tumor cells: human lung carcinoma (A549) and human breast carcinoma (MDA-MB-231) were cultured in Dulbecco's modified Eagle Medium (DMEM) with 4.5 g / L glucose, 2 mM L-glutamine and supplemented with 10% fetal bovine serum (FBS) and antibiotic mixture (100U/mL penicillin and 100µg/mL streptomycin). During the experiments the cells were kept under standard conditions: humidified atmosphere with 5% CO_2, temperature 37°C and were split every two days.

Alamar blue assay was used to determine cell viability. This technique is a commonly used method for cytotoxicity and cell viability assays. The principle of the method consists in the continuous transformation / reduction of resazurin, the Alamar Blue substance, via viable cells to resorufin, a molecule that produces a red fluorescence that can be quantified, and, thus quantitative results on cell viability and cytotoxicity are obtained. Cells ($1x10^4$ cells/ well) were grown in 96-well plates and allowed to adhere to the plate for 18-24h (overnight). After 24 h the medium was discarded, and the cells were stimulated by adding different concentration of extracts to the culture medium. A volume of 20µL of Alamar blue (10% of the medium volume contained in the well) / well was added and incubated for 3h, the following step consisting in reading the absorbance at two different wavelengths, 570 and 600 nm by using a xMark™ Microplate Spectrophotometer

Extract	Extraction yields (%)	TPC (mg GAE/g dm)	TFC (mg RE/g dm)	TFvC (mg RE/g dm)
M.S.l1	20.4	34.8±1.2	23.2±0.6	6.7±1.1
M.S.ls1	26.2	18.9±0.9	31.1±0.8	11.3±1.0
M.S.l2	12.6	26.3±1.0	22.3±0.4	5.2±1.2
M.S.ls2	13.8	16.4±1.2	39.6±1.0	10.1±0.6

Table 1
TOTAL PHENOLIC, FLAVONOID AND FLAVONOLS CONTENT FOUNDED IN THE EXTRACTS OF *Medicago sativa* (n=3, mean ± SD)

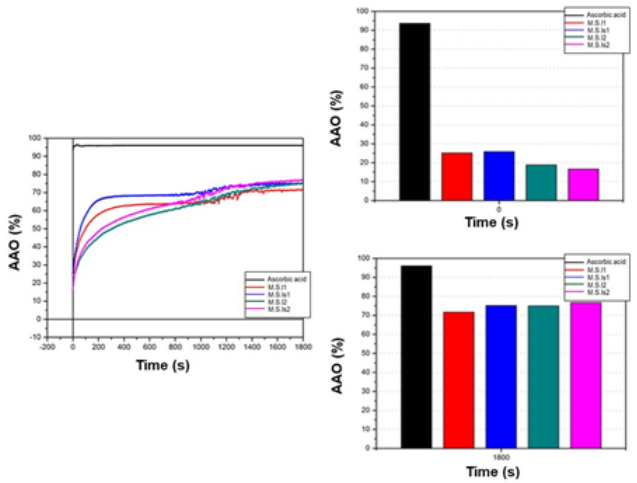


Fig. 2. Antioxidant activity of *Medicago sativa* leaves and stems total extracts over entire time interval and at initial and final moments

(Biorad) and cell viability was calculated according to the formula described in our previous studies [12].

Results and discussions

In the present study were evaluated the total phenolic, flavonoid and flavonols content, antioxidant activity and cytotoxic effects exerted by four extracts from *Medicago sativa* leaves and stems. The extraction yield percentages for the extracts from aerial parts of *Medicago sativa* using 70% ethanol and 96% ethanol by maceration techniques are presented in table 1.

The Folin-Ciocalteu method is a simple, fast and less expensive method which provide valuable primary information about the amount of phenolic compounds content from plant extracts. The highest quantity of phenols were detected in leaves extract, M.S.l1 - 34.8 mg GAE/g dm; M.S.l2 – 26.3 mg GAE/g dm, whereas leaves and stems extracts showed a lower content, M.S.ls1 - 18.9 mg GAE/g dm M.S.ls2 – 16.4 mg GAE/g dm, respectively. Extracts are rich in hydroxylated compounds, which is confirmed by the above presented results and as can be seen, larger amounts of phenolic compounds are found in extracts obtained using 70% ethanol, a proper polar solvent for this class of compounds. Alfalfa leaves and stems were particularly abundant in flavonoids and flavonols as it can be seen in the data presented in table 1. Our data are in accordance with other studies conducted in the same area [4,9]. Table 1 reveals the total phenolic, flavonoids and flavonols content of the extracts measured using the Folin–Ciocalteu method and aluminum colorimetric assay, respectively.

Figure 2 presents the AAO of the four extracts which proved to have a high AAO if we compare it to the AAO of ascorbic acid used as the reference substance. All samples showed a sharply increase of AAO in the first 200 s, after which they reach a near-balance state presenting a slow insignificant increase. The graphs show that M.S.ls1 extract possesses the highest increase followed by M.S.l1 extract. Regarding the M.S.l2 and M.S.ls2 extracts, the initial growth is slower in the first 200 s, but the whole AAO shows a steady progressive increase and at the end of the analysis time they have an AAO at least equal to the extracts discussed above which were obtained by maceration with 70% ethanol.

If at the initial time total M.S.ls1 extract exerts the highest antioxidant activity, followed by the total M.S.l1 extract, at the final moment M.S.ls2 extract showed the highest activity, followed by M.S.ls2 and M.S.ls1 with approximately equal values (fig. 3).

Cell viability

Cytotoxic effect of *Medicago sativa* extracts assessed by Alamar blue assay was expressed as percentage of viable cells (%) related to the control cells (the cells that were stimulated with the same concentration of solvent used). Alamar blue technique was designed in order to quantify the proliferation of different cell types of human or animal origin, bacteria and fungi. Despite the constant evolution of techniques in the field this method continues

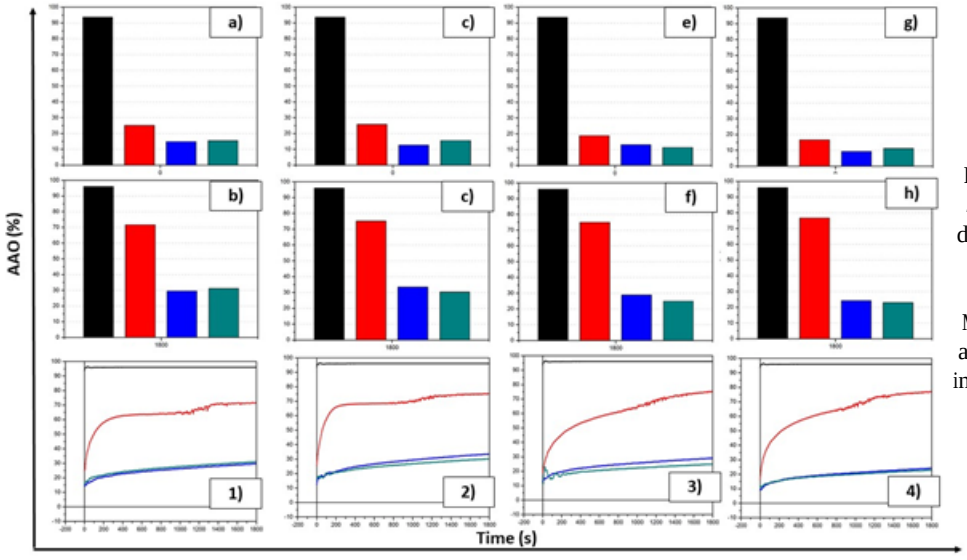
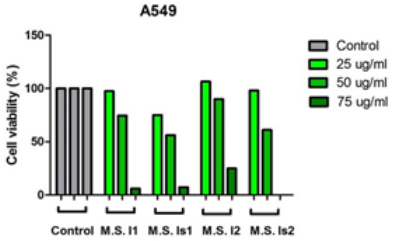


Fig. 3. Antioxidant activity of *Medicago sativa* extracts (red-total extract; blue-dilution 1:5; green-dilution 1:10): a-h) at initial moment (t=0) and at the final moment (t=1800): a,b – M.S.l1; c,d – M.S.ls1; e,f – M.S.l2; g,h – M.S.ls2. Total antioxidant activity over the entire time interval: 1 - M.S.l1; 2 – M.S.ls1; 3 – M.S.l2; 4 – M.S.ls2



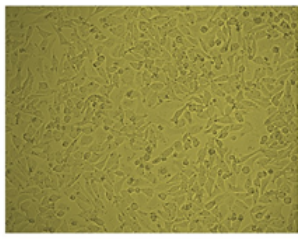
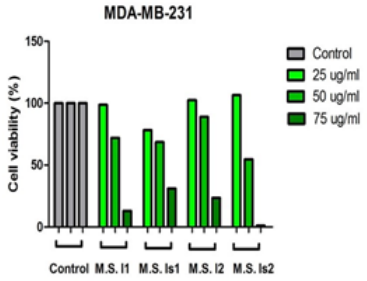


Fig. 4. The cytotoxic effect on A549 cells induced by stimulation with test samples (25, 50, and 75 µg/mL) for 24h assessed by Alamar blue assay. The results are presented as percentage of viable cells reported to control cells (unstimulated cells)



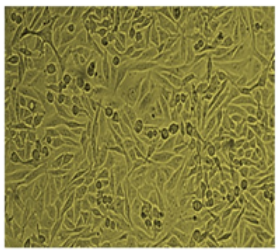


Fig. 5. The cytotoxic effect on MDA-MB-231 cells induced by stimulation with test samples (25, 50, and 75 µg/mL) for 24h assessed by Alamar blue assay. The results are presented as percentage of viable cells reported to control cells (unstimulated cells)

to be used successfully applied for the assessment of cell viability and cytotoxicity, especially for determining the efficacy and relative cytotoxicity of different chemical or natural compounds [13].

Stimulation of the tumor cells with different concentrations (25, 50, and 75 µg/mL) of samples M.S.l1, M.S.ls1, M.S.l2 and M.S.ls2 for 24 h led to a decrease of cell viability as compared to control cells (unstimulated cells), as presented in figures 4 and 5. .

The viability of the A549 cells was significantly affected at the highest concentration used (75µg/mL) for all tested extracts (fig. 4). M.S. l1 and M.S. ls1 at 25 and 50 µg/mL seemed to be more cytotoxic as compared with the M.S. l2 and M.S. ls2 (fig. 4).

In the case of human breast carcinoma – MDA-MB-231 cells, M.S. l2 and M.S. ls2 exerted a similar cytotoxic effect as in the case of A549 cells, the percentage of viable cells being lower than 1% at the highest concentration (fig. 5). M.S. l1 and M.S. ls1 stimulation was associated with a significant decrease of cells viability at 75µg/mL, effect that was lessened as compared to the one recorded for A549 cells (fig. 5).

Conclusions

In conclusion, our results indicate an increased content of phenolic and flavonoid compounds in all four extracts prepared and tested what led to a high antioxidant activity. Leaf and stem extracts proved a potent cytotoxic effect on both tumor cell lines. These results demonstrate that the extracts may have interesting potential in cancer chemoprevention and therapy.

References

1. ANTAL, D.S., CORICOVAC, D., SOICA, C.M., ARDELEAN, F., PANZARU, I., DANCIU, D., VLAIA, V., TOMA, C., Rev. Chim.Bucharest), **65**, no.9, 2014, p. 1122
2.RODRIGUES, F., PALMEIRA-DE-OLIVEIRA, A., DAS NEVES, J., SARMENTO, B., AMARAL, M.H., OLIVEIRA, M.B., Ind. Crops Prod. 49, 2013, p. 634
3. MA, Q.-G., LI, T., WEI, R.-R., LIU, W.-M., SANG, Z.-P., SONG, Z.-W., J. Agric. Food Chem., 64, 2016, p. 8138
4. KRAKOWSKA, A., RAFINSKA, K., WALCZAK, J., KOWALKOWSKI, T., BUSZEWSKI, B., J. AOAC Int. 100(6), 2017, p. 1681
5. BORA, K.S., SHARMA, A., Pharm. Biol., 49(2), 2011, p. 211
6. GATOUILLAT, G., MAGID, A.A., BERTIN, B., OKIEMY-AKELI, M.-G., MORJANI, H., LAVAUD, C., MADOULET, C., Nutr. Cancer, 66(3), 2014, p. 483
7. RAFINSKA, K., POMASTOWSKI, P., WRONA, O., GORECKI, R., BUSZEWSKI, B., Phytochem. Lett., 20, 2017, p. 520
8. MARTINEZ, R., KAPRAVELOU, G., PORRES, J.M., MELESIO, A.M., HERAS, L., CANTARERO, S., GRIBBLE, F.M., PARKER, H., ARANDA, P., LOPEZ-JURADO, M., Journal of Functional Foods 26, 2016, p. 470
9. KARIMI, E., OSKOUEIAN, E., OSKOUEIAN, A., OMIDVAR, V., HENDRA, R., NAZERAN, H., J. Med. Plants Res. 7(7), 2013, p. 290
10. ISAIA (OARCEA), A.I., CATA, A., OLAH, N.K., STEFANUT, M.N., IENASCU, I.M.C., BRATOSIN, D., POPOIU, C., Rev. Chim.(Bucharest), **67**, no.10, 2016, p. 2001
11. ROUPHAEL, Y., BERNARDI, J., CARDARELLI, M., BERNARDO, L., KANE, D., COLLA, G., LUCINI, L., J. Agric. Food Chem. 64, 2016, p. 8540
12. CORICOVAC, D.E., MOACA, E.A., PINZARU, I., CITU, C.; SOICA, C., MIHALI, C.V., PACURARIU, C., TUTELYAN, V.A., TSATSAKIS, A., DEHELEAN, C.A., Front. Pharmacol., 2017, 8, p. 154.
13. BONNIER, F., KEATING M.E., WROBEL T.P., MAJZNER K., BARANSKA M., GARCIA-MUNOZ A., BLANCO A., BYRNE H.J., Toxicol. in Vitro, 29, 2015, p. 124

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