Total phenolic content, FTIR analysis, and antiproliferative evaluation of lupin seeds harvest from western Romania

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▋ Abstract

Introduction. Lupinus spp. are herbaceous perennial flowering plants included in the Fabaceae family. Among the approximately 200 species belonging to this genre, Lupinus albus L., also known as white lupin, Lupinus angustifolius L., and narrow-leafed lupin or blue lupin, represent two of the most studied species due to their intense culinary use and potential biological activity. The intention of the study was to characterize total phenolic content, perform FTIR analysis, and antiproliferative effects against A375 human melanoma cells extracts obtained from germinated and ungerminated seeds from Lupinus albus L. and Lupinus angustifolius L.

Materials and method. Total phenolic content was assessed using the Folin-Ciocalteu colorimetric method. FTIR spectra were carried out by a Shimadzu Prestige-21 spectrometer in the range 400–4000 cm^{-1}, using KBr pellets and resolution of 4 cm^{-1}. Antiproliferative capacity was screened by employing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and scratch assay methods.

Results. The study showed increased values corresponding to total phenolic content for the germinated extracts. FTIR spectra confirmed the presence of genistein and main cinnamic acids derivatives (ferulic, caffeic, rosmarinic, and coumaric acids). All tested extracts showed weak antiproliferative potential against A375 human melanoma cells.

Conclusions. Germination increased the amount of bioactive compounds in the seeds of the two studied species of lupin. FTIR analyses provided an important fingerprint of the chemical composition.

▋ Key words

antiproliferative, FTIR, germination, polyphenols, lupin seeds

INTRODUCTION

Natural compounds represent a very important source for the discovery of new drugs with important therapeutic activity in different areas of medicine [1]. Nevertheless, research in this area shows that less than 10% of the world's biodiversity have been screened for possible biological effect, thus, many more primary and secondary plant metabolites await discovery in order to be put into service for the health benefits of the human body [2]. The *Fabaceae* family, also known under the name of legume, pea, or bean family, provides an increased number of nutraceuticals, plants used both for the nutritional value as well as for their health benefits. Generally, the seeds are used in the alimentary field due to the high amount of proteins, carbohydrates, polyunsaturated fatty acids, dietary fibre, and in the medical field due to the presence of flavonoids, isoflavonoids, poly-hydroxy acids, phytosterols, vitamins and minerals [3]. *Lupinus* spp. are herbaceous perennial flowering plants, being part of the *Fabaceae* family. Among the approximately 200 species belonging to this genre, *Lupinus albus* L., also known as white lupin, *Lupinus angustifolius* L., narrow-leafed lupin or blue lupin, represent two of the most studied species due to their intense culinary use and potential biological activity. The most important chemical constituents of the seeds represented by proteins, with percentages in the range of 36–52%, followed by fibres with quantity in the range of 30–40%, and essential oils in amounts with the range of 5–20%. Other chemical constituents worth mentioning, but present only in a concentration under 5%, include oleic and linoleic acids, flavonoids, isoflavonoids, carotenoids, polysaccharides, alkaloids. The amount of the above- mentioned constituents can vary depending on the environment, pedoclimatic and genetic conditions [4–6]. It is also very important to mention that from the point of view of the amount of protein, lupine seeds follow closely soybean seeds, and as a particular advantage, *Lupinus* spp. can be cultivated in Europe while *Glycine* spp. prefer pedoclimatic conditions in Asia [4].

Regarding the biological activity of lupin seed extracts, antioxidant activity has been reported [7]. Lampart-Szczapa

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et al., have described the antibacterial activity of lupin seeds extracts on both Gram (+) and Gram (-) strains, and concluded that it is directly correlated with the amount of total polyphenols [8]. Erdemoglu *et al.,* have shown that the alkaloid extract obtained from *Lupinus angustifolius* L. possesses significant antibacterial and antimitotic activity against *B. subtilis, S. aureus, P. aeruginosa, C. albicans and C. kruse,* and has a weak potential against *E.coli* [6]. The group of Sirtori *et al.,* have demonstrated that proteins extracted from white lupin seeds present cholesterol-lowering effects in rats, and augment the activity of the LDL receptor in the human hepatoma HepG2 cell line [9]. Also, the extracts obtained from blue lupin seeds have been reported for their cholesterol-lowering potential using an animal model of intact and ileorectal anastomosed pigs [10]. In the same way, Marchesi *et al.,* have described the hypolipidaemic and anti-atherosclerotic potential of the proteins extracted from the seeds using a rabbit model of atherosclerosis [11]. Its implications in the treatment of type 2 diabetes were assigned to conglutin [12]. Anthelminthic properties for the seeds extract where described by Dubois *et al.* [13].

The aim of this study is to characterize in terms of total phenolic content, FTIR analysis and antiproliferative effects against A375 human melanoma cells extracts obtained from germinated and ungerminated seeds from *Lupinus albus* L. and *Lupinus angustifolius* L.

MATERIALS AND METHOD

Vegetal extracts. *Lupinus albus* L. and *Lupinus angustifolius* L. seeds where obtained from the experimental culture field of Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" in Timisoara. Voucher specimens were identified and deposited in the herbarium of the University of Medicine and Pharmacy "Victor Babeş", Department of Pharmacognosy. Seeds where divided in two groups. One group was stored in standard conditions until analysis and the other group was subjected to germination, as previously described by Andor *et al.* [4]. For both germinated and ungerminated seeds, extracts were prepared using 80% ethanol (FALC LCD series ultrasonic bath, for 10 min at 40 kHz and 28 °C). Extraction was repeated three times. A rotary evaporator was used to eliminate the solvent. To simplify the presentation of results, the following letters where assigned: A – ungerminated *Lupinus. albus* L. seeds extract; B – ungerminated *Lupinus angustifolius* L. seeds extract; C – germinated *Lupinus. albus* L. seeds extract; D – germinated *Lupinus angustifolius* L. seeds extract.

Total phenolic content (TP). Determined using the Folin-Ciocalteu colorimetric method. Seed extracts were diluted (1:4, v/v) with water, treated with 20 ml methanol: water (50:50, v/v) and maintained at room temperature for one hour. The samples were centrifuged (10 minutes at 450 rot/min) and 0.5 ml of supernatant was treated with 1.25 ml Folin-Ciocalteu reagent diluted 1:10 with water. The samples were incubated for 5 minutes at room temperature and 1 ml Na_2CO_3 60g/l was added. After 30 min of incubation at 50 °C, the absorbance of samples was measured at 760 nm. Linearity was obtained between 5–50 μg/ml. Results were expressed as mg of gallic acid equivalents per 100 g fresh weight (FW) sample (mg GAE/100g FW).

Fourier transform infrared spectroscopy (FTIR). Samples were also characterized by means of infrared spectroscopy. FTIR spectra were carried out using a Shimadzu Prestige-21 spectrometer in the range 400–4000 cm^{-1}, using KBr pellets and resolution of 4 cm^{-1}.

MTT. A375 human melanoma cells were seeded onto a 96-well culture plate at a cellular density of 1×10^4 cells/well and attached to the bottom of the well overnight. After 24 hours, 100 μL of new medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA) and 50 and 150 μg/ml of the tested substances (dissolved in dimethyl sulfoxide – DMSO; Sigma-Aldrich Company), were added and incubated for 72 h; the medium was supplemented with 10% foetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 1% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell, Heidelberg, Germany). The cells were then assayed by the addition of 10 μL of 5 mg/mL MTT solution from the MTT-based *in vitro* toxicology assay kit (Tox-1; Sigma-Aldrich). The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 3 h contact period. The precipitated crystals were dissolved in 100 μL of lysis solution (Sigma-Aldrich). The reduced MTT was spectrophotometrically analyzed at 590 and 655 nm, using a microplate reader (Bio-Rad, Hercules, CA, USA). Stock solutions of the tested substances (10 mg/ml) were prepared in DMSO.

Scratch assay. The migratory ability of selected tumour cells (A375 human melanoma cells and MDA-MB-468 breast carcinoma cells) was examined *in vitro* by using the scratch assay technique. A number of 2×10^5 cells/well was cultivated in 12-well plates for 48 h prior to the experiment. A sterile pipette tip was used to draw scratches in well-defined zones of the cells monolayer (confluence of 80–90%). Cells that detached as a result of the procedure were removed by washing with PBS before stimulation. The cells were stimulated with different selected extracts (50 and 150 μg/ml) following solubilization in DMSO (stock solution 10 mg/ml). Images of the cells in culture were acquired at the starting point of the experiment, and after 4 and 24 h using an Optika Microscopes Optikam Pro Cool 5 and Optika View software.

Statistics. The Prism software package (Graph Pad Prism 4.03 for Windows) was used for presentation of the experimental data. Results are presented as mean ± SD. Two way ANOVA was applied to evaluate statistical significance (*, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$).

RESULTS

Total phenolic content of the selected extracts expressed as mg gallic acid equivalents (GAE) per 100 g sample are shown in Figure 1. It can be observed that the two analyzed lupines species extracts present similar values in terms of total phenolic amount, and that the amount is increased after germination.

The results of the FTIR analysis for samples A, B, C and D are presented in Figure 2. All spectra were recorded between 400 – 4,000 cm^{-1}.
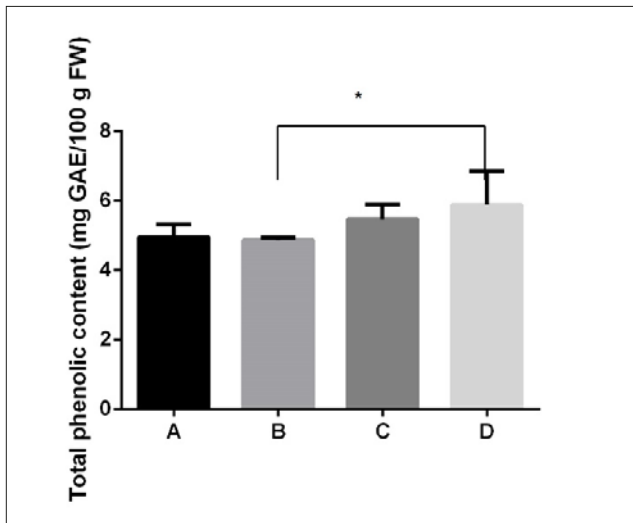
Figure 1. Total phenolic content of the selected extracts expressed as mg gallic acid equivalents per 100 g FW sample (unpaired t test, t=0,0113)

As it can be observed from the FTIR spectra, all samples present the same absorbance bands, the differences between them consisting in the value of the transmittance and in the appearance of the bands (width, sharpness). These findings are predictable since the samples are four extracts obtained from germinated and ungerminated seeds from two different species of lupin, as described in the materials and method section. According to the literature presented in the discussion section, due to the assigned groups to the vibrational mode it can noted that the analyzed samples show correspondence in the occurrence of the main groups. The

assigned groups (due to the f molecules vibration) belong to compounds identified by means of HPLC (isoflavone, main cinnamic acids derivatives).

The MTT proliferation assay showed that at the tested concentrations, using an incubation time of 72 h, the extracts of both lupin species presented weak (inhibition
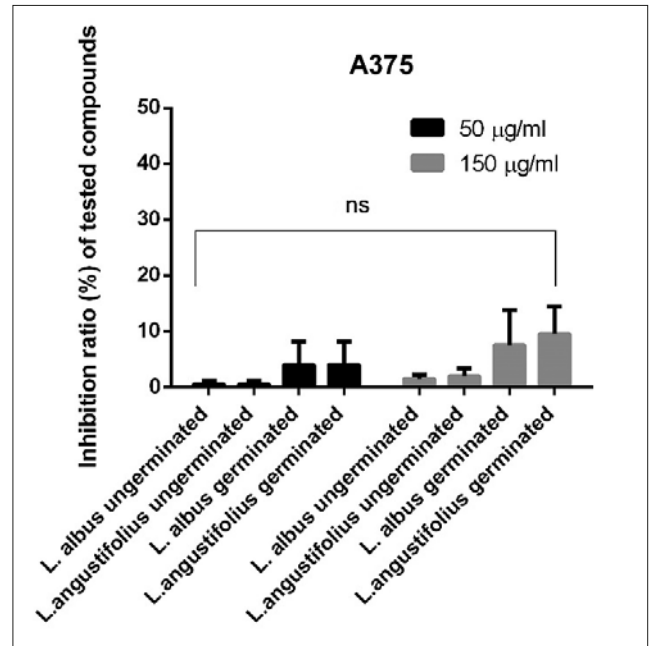

Figure 3. Antiproliferative effect of *Lupinus albus* L. and *Lupinus angustifolius* L. ungerminated and germinated seeds extract against A375 human melanoma cell line
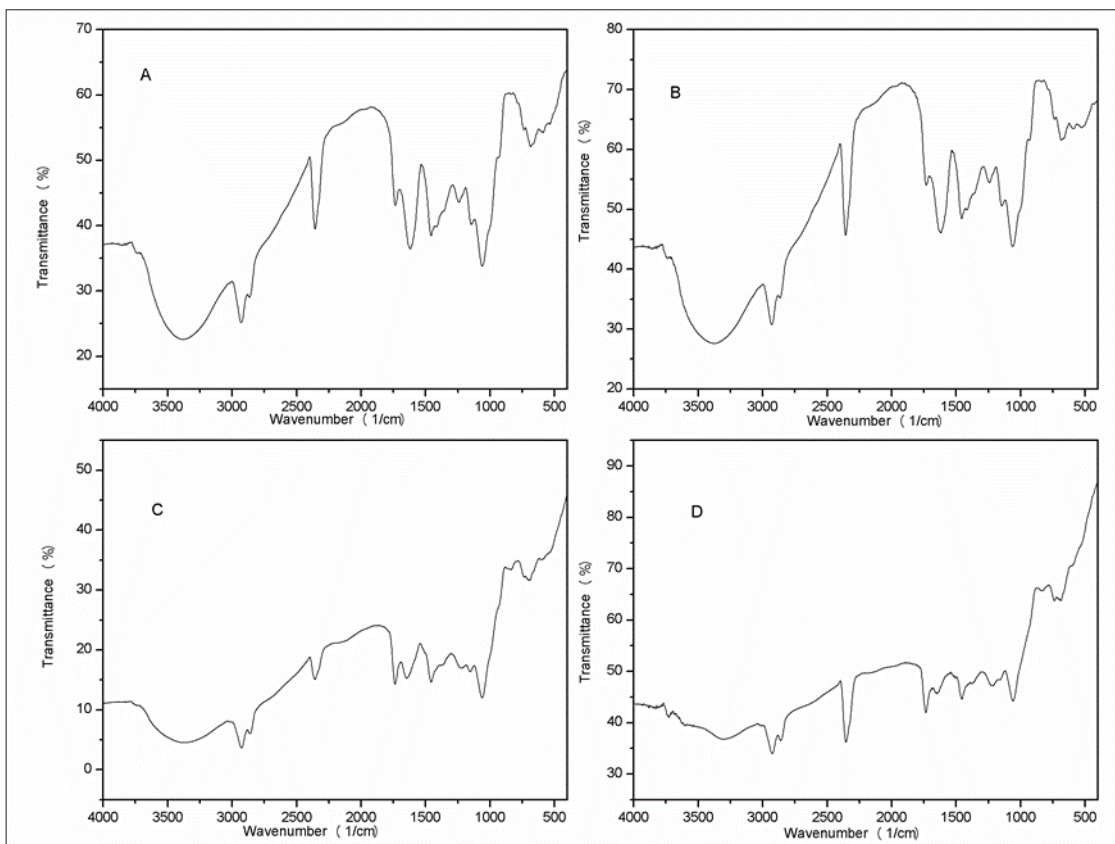

Figure 2. FTIR spectra for sample A-D

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ratio under 20%) antiproliferative activity against A375 human melanoma cells. As a particular observation, the antiproliferative activity was slightly increased in the case of both germinated seeds extracts, thus being proof of the augmentation of the concentration of the bioactive compounds, but still not enough to proclaim a significant antiproliferative effect (Fig. 3).

The scratch assay strengthens the findings about the weak antiproliferative effect of the sample selected cell lines. Cell migration was not inhibited for none of the two tested cell lines after incubation with the highest tested concentration of the four samples, namely 150 µg/ml. Again in case of incubation with germinated seeds extract migration seems to be slightly diminished compared to the groups incubated with ungerminated seeds extract (Fig. 4).

DISCUSSION

The amount of polyphenols in vegetal products reflects their antioxidant capacity, and consequently, their potential in preventing various oxidative stress associated diseases. Given their multiple benefits there has been much interest in the study of this plant's secondary metabolites [14, 15]. The amount of polyphenols in a vegetal product varies depending on the extraction method involved [16]. Wang *et al.* showed that the amount of polyphenols for *Lupinus albus* L. seeds varies between 444.4 – 1661.2 mg/100 g of vegetal product, whereas for *Lupinus angustifolius* L. seeds it varies between 535.1 – 578.4 mg/100 g [17]; the total polyphenols in the extracts of ungerminated seeds of the two *Lupinus* spp. detected during the present investigation are consistent with previous studies [18]. Germination of different legumes increases the amount of bioactive compounds, thus providing useful products for nutraceutical applications [19]. Duenas
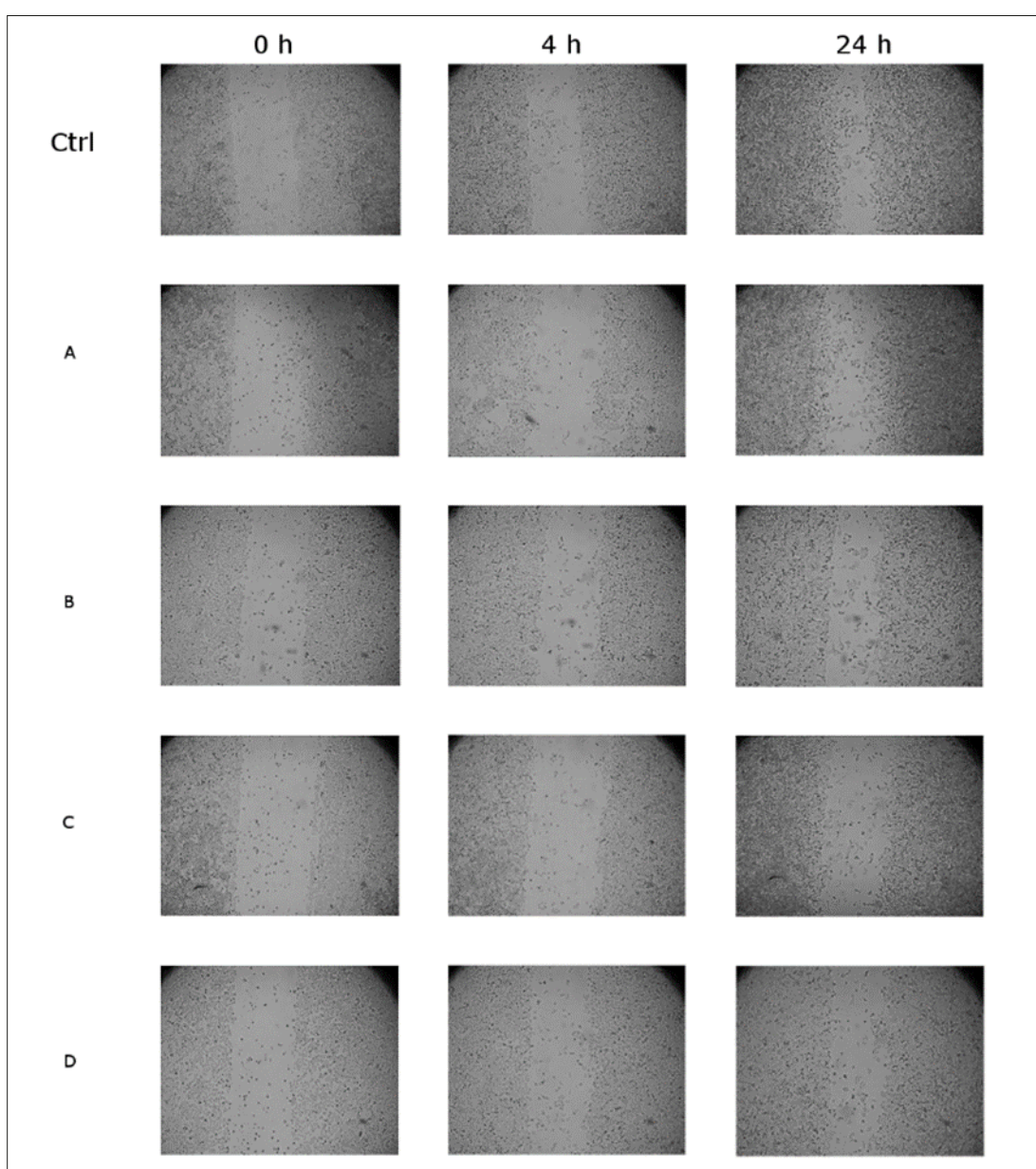

Figure 4. Anti-migration capacity of *Lupinus albus* L. and *Lupinus angustifolius* L. ungerminated and germinated seeds extract against A375 human melanoma cell line

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et al. have depicted germination as a method of increasing the polyphenol content as well as antioxidant activity for *Lupinus angustifolius* L. [20]. The results from the current study are in agreement with previous reports from the literature, as explained in the Results section, in which it was possible to detect higher amounts of polyphenols in the germinated seeds of the two *Lupinus* sp.

Some of the main phytochemicals present in the analyzed samples were established by means of HPLC in a previous study [4]. The targeted chemical compounds identified by HPLC were the isoflavone genistein, together with caffeic coumaric, ferulic, and rosmarinic acids. The vibrational spectrum of any molecule has a distinctive property and is characteristic for that molecule. In this case, each analyzed sample had more than one compound, which make it difficult to correlate the vibrational band from the spectra with its correspondent compound. The disadvantage of infrared spectroscopy is the overlapping of the bands, resulting in complicated attribution of the identified group; this aspect is emphasized when the analyzed sample is formed by a mixture of compounds. The FTIR analysis has its own limitation [21], it can help elucidate the structure of a known compound but cannot establish with certainty the identity in mixtures of compounds.

According to [22], the region for functional groups is between 4,000–1,300 cm^{-1}. Structures containing: carbonyl groups are located in the 1,850–1,540 cm^{-1} region, strong skeletal bands for aromatics in the 1,600–1,300 cm^{-1} region (resulted in a ring structure from stretching of the carbon-carbon bonds), aliphatic structures are located in the region 909–650 cm^{-1}. Also, the fingerprint region is mentioned, which is not to be considered between 1,300–909 cm^{-1}.

In the book by B. Stuart [23], the author described that the alifatic compounds type methylene groups present stretching at 2,930 cm^{-1} (asymmetric) and at 2850 cm^{-1} (symetric); the =C-H binding between 700–300 cm^{-1}; combination bands – overtone in the region 2,000–1,700 cm^{-1}. For the aromatic compounds, C-H streching are in the 3,100–3,000 cm^{-1} area; in the 2,000–1,700 cm^{-1} region there are located a weak combination, overtone bands, and substitution of the benzene ring; 1,650–1430 cm^{-1} is the region for skeletal vibration (C=C streching); in-plane binding of C-H is located between 1,275–1,000 cm^{-1}; out-of plane binding appear in the region 900–690 cm^{-1}.

A comprehesive study about trans-cinnamic acids which brings together data obtained from both FT-Raman and FTIR spectra offers important information regarding the characteristic absorptions bands for caffeic, coumaric, ferulic, and rosmarinic acids [24]. At values of 3,000 cm^{-1} the vibration are attributed to stretching the C-H ring and stretching =C-H; around 1,600 cm^{-1} are located the bands for symmetric and asymmetric stretching in C=O; between 1,430 -1,580 cm^{-1} are the bands corresponding to stretching vibration in the C-C ring; in the range 1,030–1,180 cm^{-1} are located the bands in plane deformation of the C-H ring. At 1,267 cm^{-1} is presented the position for in-plane deformation of =C-H, at 683 cm^{-1} COOH scissoring and at 769 cm^{-1} out of plane deformation of the C-H ring [24]. The out-of plane, in-plane and out-of plane ring deformation are also presented. It is important to mention that in a mixture such as in the presented case, the extracts, a multitude of bands that overlaps and perhaps converge into a broader band, or could shift to higher wavenumber, can be detected on the spectra.

Crupi at al. [25] stress that in the range 4,000–2,000 cm^{-1} genistein presents an absorption band at approximately 3,405 cm^{-1}, normally attributed to the O-H stretching vibration, overlapped with a band located at 3,084 cm^{-1} attributed to C-H stretching vibration. In the presented case, the broad band located at over 3,000 cm^{-1} could be assigned for this kind of vibrational behaviour. Usually, the hydroxyl groups are present in the range 3,100–3,700 cm^{-1} [26]; however, in mixtures and complex molecules these groups are overlapped, resulting in very broad bands, that lead to a difficult correlation of the spectral profile.

The anti-proliferative and anti-migratory effects are frequently discussed together in order to get an idea of a possible chemopreventive effect of the tested sample [27]. The literature is very poor in reports regarding results about the anti-proliferative, respective anti-migratory effect of extracts obtained from lupin seeds which resulted in the presented study to perform screening regarding the chemopreventive potential of this vegetal product. As can be observed from the results section, none of these activities are notable; thus results of this study are not helpful for providing data about a significant biological activity to scientists in the field with similar question to those posed by the authors of this study.

However, it can be clearly seen that the anti-proliferative and anti-migratory effects are increased for the germinated samples, compared to ungerminated ones. Germination is a very good and accepted method used for the augmentation of the amount of the biological active substances [28]. In a similar approach, Andor et al., tested the antiproliferative activity of germinated and ungerminated extracts of lupines seeds against breast (MCF7, MDA-MB-231 cell lines) ovarian (A2780cell line), and cervical cancer (SiHa cell line), and described a similar decreased activity, with the observation that germination slightly increased the biological effect [4].

CONCLUSIONS

Extracts obtained from germinated seeds contain an increased amount of polyphenols in comparison to extracts obtained from ungerminated seeds. FTIR spectra confirmed the presence of genistein and main cinnamic acids derivatives (ferulic, caffeic, rosmarinic, and coumaric acids). All tested extracts showed weak antiproliferative potential against A375 human melanoma cell line.

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