FLAXSEED CAKE – A SUSTAINABLE SOURCE OF ANTIOXIDANT AND ANTIBACTERIAL EXTRACTS

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Flaxseed cake, a by-product of the oil cold pressing technology, was subjected to extraction and acidic hydrolysis to obtain extracts rich in polyphenols and especially in lignans. The second step was to assess the extracts in order to evaluate their potential to be used as additives in the food and cosmetics industries. The identification and quantification of secoisolariciresinol (SECO), matairesinol (MATA) and lariciresinol (LARI) in the said extracts was accomplished using the HPLC method. The total polyphenol content, radical scavenging activity and antimicrobial activity were determined. The total polyphenol content for the samples extracted with 60% ethanol, hydrolyzed at 80 °C, was comparable to that of the extracts obtained from spices or medicinal plants. The radical scavenging ability of the extract correlates well with the total polyphenol content and is comparable to that of common synthetic phenolic antioxidants used in foods and cosmetics. Flaxseed cake extracts also contain up to 7.08 mg SECO/L, 0.06 mg MATA/L and 0.03 mg LARI/L. Both crude and hydrolysed extracts exhibited antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The ability to extract agro fine chemicals from flaxseed cake provides the flaxseed industry with value-added co-products.

Keywords: flaxseed cake, lignans, polyphenols, HPLC, antimicrobial activity

INTRODUCTION

Flaxseed cake, a by-product that results from flaxseed cold pressing, is an important source of omega 3 and omega 6 rich oils. What is more, instead of blending it into fodder, it could be valued as a source of agro fine chemicals. Removing oil from flaxseeds prior to extraction doubles the amount of phenolic acids and lignans in the obtained extracts.[1]

Flaxseed cake represents a cheap, raw material for phenolic and especially lignans rich extracts, and a partially predefatted source, leading to decreased extraction costs. The importance of lignans and phenol extraction roots in their remarkable biological activities as antioxidants, antimicrobial and anticarcinogenic compounds.[2,3,4]

Secoisolariciresinol diglucoside (SDG), the major flaxseed lignin, is 75-800 times more abundant in flaxseeds than in other food sources.[5]

Along with its derivative, secoisolariciresinol (SECO), SDG is the main precursor of the mammalian lignans (enterodiol and enterolactone), being known as an efficient chemopreventive for breast, prostate and ovary cancer.[6,7] Moreover, several other studies have demonstrated that flaxseed lignans decrease serum cholesterol and blood pressure, prevent diabetes and cardiovascular diseases.[8,9,10] SDG is present in the flaxseeds as ester linked oligomers, as well as polymers consisting of SDG and 3-hydroxy 3-methyl glutaric acid (HMGA).[14,15,16,22]

This oligomeric lignan derivative is readily soluble in aqueous methanol or ethanol. However, alkaline hydrolysis is subsequently needed to release SDG, or acid hydrolysis to release secoisolariciresinol mono-glucoside (SMG), SECO and even anhydro-SECO. It all depends on

the acid concentration, hydrolysis duration and temperature.[11,12,13,14,15,16,17]

Several methods are known to identify and quantify the lignans present in flaxseed extracts. To name a few, we could mention GC-MS[14] and HPLC.[16] The latter is the most common method for the analysis of lignans in flaxseeds, as it is fast, simple and convenient.

Over the years, there has been a tendency to replace common synthetic phenolic antioxidants used in foods and cosmetics – including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) – with natural phenolic antioxidants extracted from plants.[18,19,20] Such is the case of tocopherols and rosemary extracts.[18,21]

The aim of this study is to analyse the potential of flaxseed cake as a cheap, natural, raw material for lignan rich polyphenolic extracts. Optimizing extraction conditions will also be discussed here. The obtained food-grade flaxseed cake extract will be evaluated and compared to other phenolic extracts from spices and medicinal plants, as it is important for future applications in nutraceuticals, food products and cosmetics. This will help improve those products' quality, safety and prolong their shelf life.

EXPERIMENTAL

Materials

Flaxseeds belonging to a *Linum usitatissimum* Romanian cultivar, called "Cosmin" were obtained from Research & Agricultural Development Institute, Livada, in Satu Mare County, Romania, were sown and harvested in 2011. This cultivar is known for producing quality textile fibres with good yield, its seeds being also an important provider of omega 3, omega 6 rich oils. Flaxseed cake is a by-product of oil cold pressing technology, which separates most of the omega 3, omega 6 rich oils from the vegetal matrix.

Reagents, solvents and standards

HPLC standards were purchased from Sigma Aldrich (Fluka, Switzerland), while acetic acid, hexane, and methanol (HPLC grade) were purchased from Merck (Germany). All of the other reagents and solvents used in the experiments were of adequate analytical grade and were obtained from Sigma Aldrich (Fluka, Switzerland), Merck (Darmstadt, Germany) and Chimreactiv (Romania).

Microbiological media (tryptic soy agar, Müller-Hinton agar and Sabouraud dextrose agar) were purchased from Roth GMBH Co KG (Germany). Discs impregnated with antibacterial and antifungal substances, as well as the discs dispenser, were purchased from Oxoid Ltd. (UK): Gentamicin (10 µg/disc), Ofloxacin (5 µg/disc), Amikacin (30 µg/disc), Kanamycin (30 µg/disc), Cefuroxime (30 µg/disc), Erythromycin (15 µg/disc) and Nystatin (100 µg/disc). The McFarland Standard for turbidity was purchased from GrantBio Ltd. (UK). Reference bacterial strains, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida parapsilosis* ATCC 22019, were purchased from Bio-Rad Laboratories (US).

Instruments

All spectrophotometric analyses were performed using a UV-VIS double beam spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany). HPLC analysis was conducted on a Shimadzu (SP-20AV) coupled with a UV detector (SPD-20AV).

Preparing the flaxseed cake for extraction

Flaxseed cake (still containing 17% oil) was ground with a hand mill (Salzburger Model MH 4) to 1 mm particle size, and then dried at 80 °C for 1 hour until a constant mass was reached. 20 g of flaxseed powder obtained from the cake was subjected to yet another deffating step, using 85 mL n-hexane, for 2 hours, in a Gerhardt Soxtherm Multistat/SX PC extractor. The defatted flaxseed material was dried at room temperature for 24 hours in order to remove the remaining n-hexane.

Extraction procedures

Based on the previously reported extraction conditions,[14,16,17,22] an experimental design was created to find the optimal conditions for extraction and acidic hydrolysis. The combined effects of the concentration of the ethanolic solution, extraction time and hydrolysis temperature upon the yield of polyphenols and especially lignans were observed. The full factorial experimental design involved different ethanolic concentrations (100%, 80%, 60%), different extraction times (3 h, 4 h) and hydrolysis temperatures (60 °C, 80 °C) for 3 replicates.

Aiming to extract polar molecules of phenolic compounds and most of the lignans, a polar complex solvent was chosen: a mixture of ethanol (which is a low-polar solvent) and water (a strong polar solvent).[3]

15 g of prepared flaxseed cake was immersed in 100 mL ethanolic solution for 3-4 hours for extraction under continuous magnetic stirring. Due to time-temperature correlation, the chosen temperature for the solvent was 60 °C.[17]

Acid hydrolysis of crude extracts

In order to release the lignans as monomer aglycones (e.g. SECO), the crude extracts were subjected to acid hydrolysis. Hydrochloric acid was added to 10 mL of crude extract to a final concentration of 2 M. The mixture was then

maintained at 60-80 °C for 2 hours, under continuous magnetic stirring, followed by neutralization with 5 M NaOH. The resulting hydrolyzed extracts were filtered through a 0.45 µm filter membrane (PSF: PVDF) and prepared for HPLC analysis.

Spectrophotometric analysis

For fingerprinting extracts, 1 mL of both hydrolysed and crude extracts was diluted to 25 mL with distilled water. A 3 mL aliquot was measured in quartz cells (10 mm light path) at 210-400 nm in a UV-VIS spectrophotometer against a blank. The data were recorded using Win ASPECT version 2.2.1.0 software.

The amount of total phenolics for each crude and hydrolyzed extracts, expressed as equivalents of gallic acid (GAE), was determined with the help of the Folin-Ciocalteu method, as described previously.[23]

The DPPH radical-scavenging activity was assessed using an improved spectrophotometric method.[24] The DPPH was dissolved in ethanol (0.2 mM) and 3 mL of the resulting solution was mixed with a 0.1 mL sample (20 mg/mL). Absorbance was recorded at 517 nm after 1-hour incubation in a dark room. Positive controls containing 2.5 to 50 mg/L gallic acid in ethanol were used as reference. Each experiment was repeated 3 times. Inhibition of the DPPH stable free radical was calculated with Eq. (1):

$$\%\,Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} x100 \quad (1)$$

where $Abs_{control}$ is absorbance of 0.2 mM DPPH in ethanol, Abs_{sample} is absorbance of 0.2 mM DPPH + extract.

HPLC analysis

SECO, MATA and LARI concentrations were determined by the HPLC method adapted after Yuan *et al*.[15] The separation was performed on a 5 µm ZORBAX SB-C18 column (150 x 4.6 mm i.d.) at room temperature under a flow rate of 1.2 mL/min, injection volume of 20 µL. A binary solvent mixture, consisting of 1% acetic acid in water as solvent A and methanol as solvent B, was used. The analysis was performed using the following linear gradient of A-B: 0 min, 10:90; 40 min, 95:5, 45 min, 10:90 (total run time 55 min).[15] The detecting λ was set at 280 nm. Calibration curves were obtained using MATA, SECO, LARI standard methanolic solutions of known concentrations between 10-200 µg/mL. HPLC software LC Solution Version 1.22 SP1 was used for integration and automatic determination of concentration in the samples.

Antibacterial activity testing

Bacterial strains, cultured on tryptic soy agar (TSA) pH 7.3, under aerobic conditions for 24 h at 37 °C and *C. parapsilosis* on Sabouraud 2% (m/v) glucose agar for 48 h at 37 °C were used as inocula. The antimicrobial activity was estimated using a disk agar diffusion test.[25,26] 1 mL inocula (1.5×10^8/mL) of bacterial cells and yeast-like fungal blastospores in sterile physiological saline solutions were inoculated into Petri dishes (Ø = 9 cm) on Müller-Hinton agar (pH 7.3) for bacterial species and Sabouraud-agar (pH 6.5) for yeast-like fungi. Sterile paper discs, impregnated with 20 µL (20 mg/mL) of both crude and hydrolyzed extracts, were aseptically applied on the surface of inoculated plates. Control discs (with antibacterial substances) and blank discs (with solvent) were used. After incubating plates for 20 h at 37 °C for bacterial species and for 48 h at 37 °C for *C. Parapsilosis*, inhibition areas were measured as diameters of clear zones around the discs, expressed in mm.

Statistical analysis

All analyses were performed in triplicate, unless otherwise specified. Data are presented as mean values ± standard deviation. Statistical analysis was performed with the GraphPad Prism 5 software (Version 5.00 for Windows).

RESULTS AND DISCUSSION

Spectrophotometric analysis

UV-Vis spectra of extracts were measured by scanning wavelength from 210 to 400 nm. Fig. 1 shows the UV spectra of the extract obtained with 60% ethanol, hydrolyzed at 80 °C, with maximum adsorption between 210-235 nm and 265-300 nm, which confirms the presence of phenolic acids, flavonoids.[13,20] The resultant specific maximum adsorption range also implies that lignans and their derivatives are present in the extracts, as they exhibited UV absorption between 270-290 nm.[27]

The total amount of polyphenols varied in both crude and hydrolyzed extracts, depending on the ethanolic concentration of the solvent, the extraction time and the temperature during hydrolysis (Fig. 2 and Fig. 3). Total polyphenol content of the extracts increased as the ethanolic concentration of the solvent solutions decreased (from 206 mg GAE/L for 100% ethanolic extract to 1115 mg GAE/L for 60% ethanolic extract). This finding can be explained by the solvent reaching optimum polarity, since adding water to ethanol leads to an increase in solvent solution polarity. Apart from polarity, another important reason for using ethanolic solutions as solvents is that we intended to harness the obtained extracts for food-grade applications.

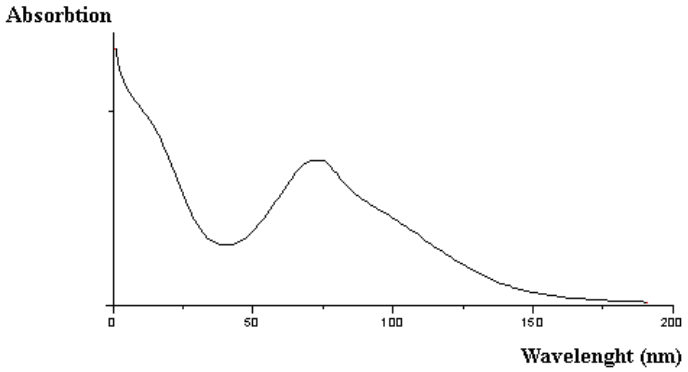

Figure 1: UV-VIS spectra of flaxseed cake extract (obtained with 60% ethanol, for 4 hours, hydrolysed at 60 °C, for 2 hours)

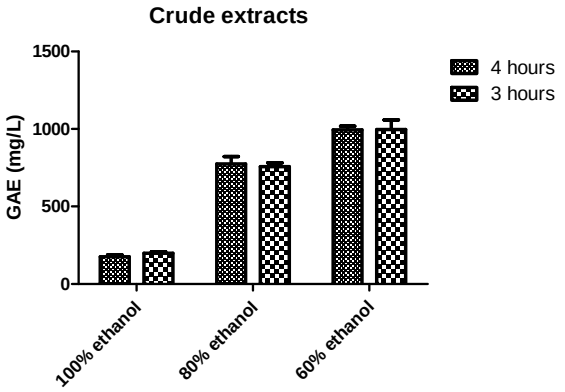

Figure 2: Total polyphenols content of crude flaxseed cake extracts ($p<0.0001$)

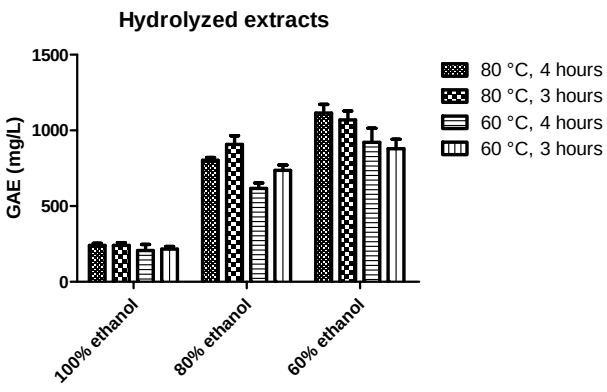

Figure 3: Total polyphenols content of hydrolyzed flaxseed cake extracts ($p<0.0001$)

As shown in Fig. 2, the total polyphenols content of the extracts obtained after 4 hours being almost equal to that of the extracts obtained after 3 hours, there is no need to prolong the extraction time by 25%, as the extraction yields are almost identical.

Total polyphenols content of the extracts hydrolyzed at 80 °C is higher by 11-30% than that of the extracts hydrolyzed at 60 °C for all the extracts obtained, irrespective of the extraction conditions (Fig. 3). These results agree with those reported by Yuan *et al*.[15] The total polyphenols content of flaxseed cake extracts is also comparable to that of other spice and medicinal plant extracts, as depicted in Table 1.

DPPH radical-scavenging activity

Crude and hydrolyzed extracts obtained both with 60% and 80% ethanol exhibited a remarkable radical scavenging ability between 21.22 and 72.95%, correlating well ($R^2 = 0.9004$) to their polyphenolic content (Table 2). The samples extracted using 100% ethanol were ineffective in scavenging the DPPH stable free radical, this finding being related to their low total polyphenols content (206-241 mg GAE/L).

Comparing the radical scavenging ability of the crude extracts to that of the hydrolyzed ones, we noticed that it doubles upon hydrolysis, probably due to disruption of linkages and deglycosylation of the phenolic compounds. These findings are sustained by many authors who have reported that phenolic compounds exhibit higher antioxidant activity as aglycones than the corresponding glycosides do, due to steric hindrance by carbohydrate moiety.[31,32,33]

Radical scavenging activities of flaxseed extracts are comparable to those of other medicinal plant and spice extracts and synthetic antioxidants (BHT, BHA) used in food products (Table 3).

Table 1
Comparative total polyphenols content of flaxseed cake extract and spice or medicinal plant extracts

Source (extract)	Total polyphenols (mg GAE/g)	Reference
Linum usitatissimum (cake)	0.743*	This work
Linum usitatissimum (seeds)	0.8	[28]
Armoracia rusticana	0.9	[28]
Pisum sativum	0.4	[28]
Daucus carota	0.6	[28]
Livisticum officinale	0.72	[29]
Carum carvi	0.7	[29]
Archangelica officinalis	0.29	[29]
Ocimum basilicum	1.38	[30]

* Calculated value (from mg GAE/L to mg GAE/g) for the extract obtained in 4 h extraction with 60% ethanol, hydrolyzed at 80 °C

Table 2
Total polyphenols content and radical scavenging ability of flaxseed cake extracts

Solvent concentration	Extracts characteristics	Type of extract			
		4 hour extraction		3 hour extraction	
		Hydrolysis at 80 °C	Hydrolysis at 60 °C	Hydrolysis at 80 °C	Hydrolysis at 60 °C
100% ethanol	Total polyphenols, mg GAE/L	241±13	206±40	240±19	216±16
	Inhibition, %	-13±7	-26±4.69	-13±0.92	-12±6
		4 hours		3 hours	
		Hydrolysis at 80 °C	Hydrolysis at 60 °C	Hydrolysis at 80 °C	Hydrolysis at 60 °C
80% ethanol	Total polyphenols, mg GAE/L	803±19	617±35	908±57	737±34
	Inhibition, %	59±3.09	51±4.89	61±8	41±7
		4 hours		3 hours	
		Hydrolysis at 80 °C	Hydrolysis at 60 °C	Hydrolysis at 80 °C	Hydrolysis at 60 °C
60% ethanol	Total polyphenols, mg GAE/L	1115±56	921±92	1069±59	879±63
	Inhibition, %	73±7	62±2.75	65±5	51±5

Results are expressed as mean ± SD (n=3)

HPLC analysis

The unhydrolyzed extracts prepared from flaxseed cake analyzed by HPLC showed no evidence of SECO, MATA, LARI, which suggests that those aglycones do not exist in free form in flaxseed, but rather as ester linked components of polar complexity. Therefore, a preliminary acid hydrolysis is necessary in order to release the corresponding aglycones successively from the oligomeric chain.[22]

The samples obtained upon hydrolysis (2M HCl) for 2 hours at 80 °C showed an increased antioxidant activity, compared to those hydrolyzed at 60 °C. This was despite the fact that SECO, MATA and LARI content decreased upon raising hydrolysis temperature. It may have been due to an advanced hydrolysis leading to complete deglucosylation of lignans. This in turn resulted in higher amounts of anhydro-SECO and other aglycones, which exhibited an increase in

antioxidant activity as compared to SDG, SMG and SECO.[15,31,33]

Table 3
Comparative scavenging ability of flaxseed cake extracts and spice and medicinal plant extracts

Source (extract)	Inhibition (%)	Reference
Linum usitatissimum (cake)	72.95	This work
Linum usitatissimum (seeds)	87.5	[34]
Salvia officinalis	41.2	[29]
Humulus lupulus	83.2	[29]
Echinaceea purpureea	75.0	[29]
Silybum marianum	34.3	[29]
Malus pumila (Punakaneli)	35.2	[28]
Rosa sp.	28.4	[28]
Andrographis paniculata	58.02	[35]
Cymbopogon citratus (Stapf)	81.69	[35]
BHT	75.6	[41]
BHA	60.8	[41]

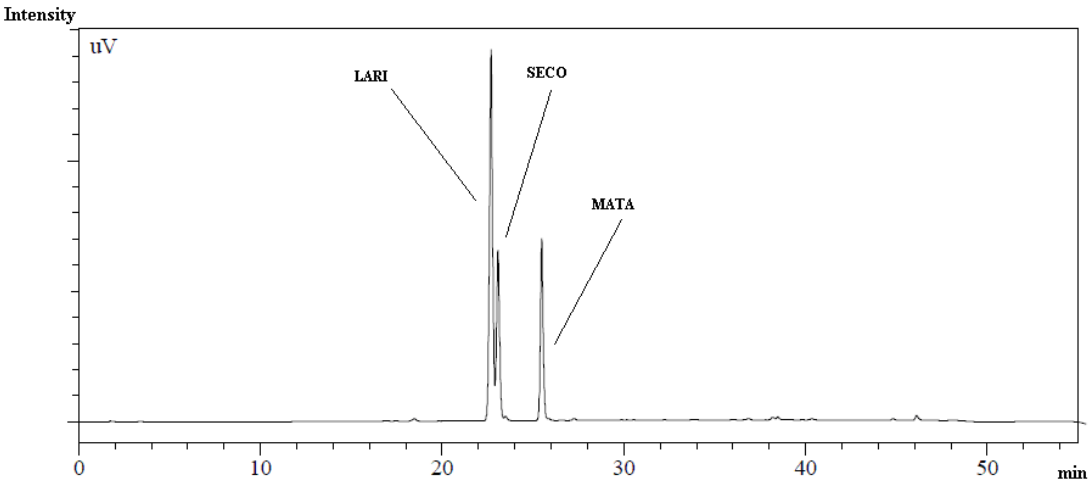

Figure 4: Chromatographic profile of standard

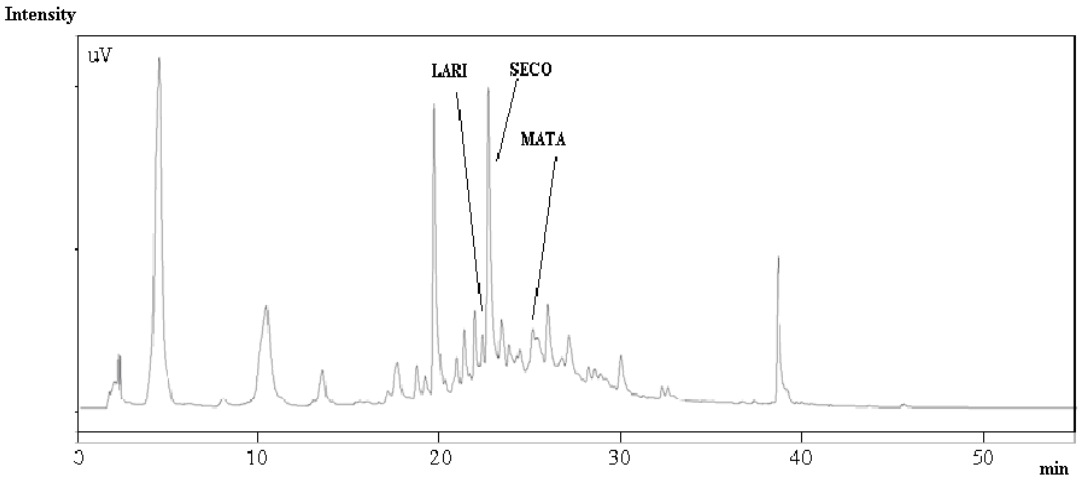

Figure 5: Chromatographic profile of flaxseed cake extract (obtained with 60% ethanol, for 4 hours, hydrolysed at 60 °C, for 2 hours)

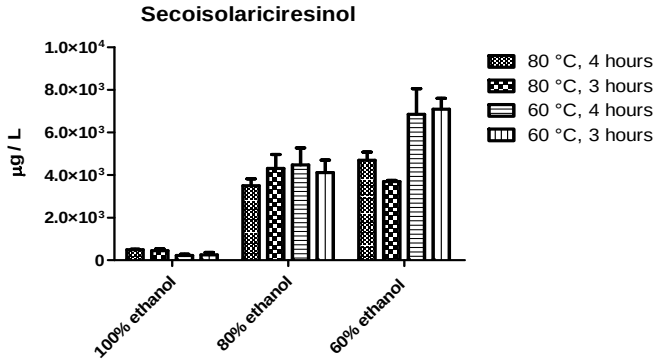

Figure 6: SECO content of flaxseed cake extracts ($p<0.0001$)

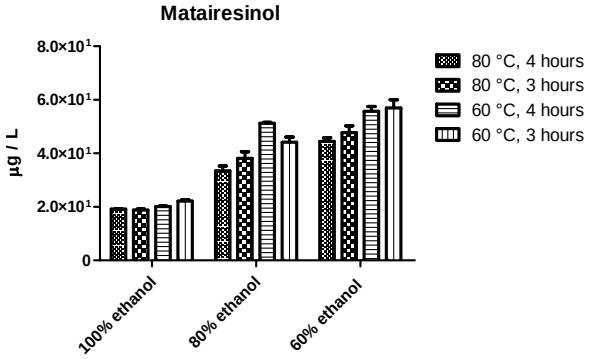

Figure 7: MATA content of flaxseed cake extracts ($p<0.0001$)

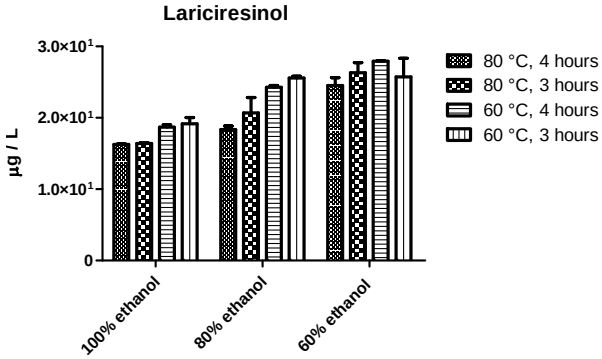

Figure 8: LARI content of flaxseed cake extracts ($p<0.0001$)

What is more, taking into consideration the fact that total polyphenols concentrations are also higher for all the extracts hydrolysed at 80 °C, we recommend this hydrolysis temperature in order to comply with our goals.

Similar to total polyphenols content, SECO and LARI concentrations of the extracts rise, as the ethanolic concentration of the solvent decreases from 0.22 mg SECO/L for 100% ethanolic extract to 7.08 mg SECO/L for 60% ethanolic extract, and from 0.02 mg LARI/L for 100% ethanolic extract to 0.03 mg LARI/L for 60% ethanolic extract (Figure 6 and Figure 8). Instead, the highest MATA content (0.06) corresponds to the sample extracted with 80% ethanolic solution (Figure 7).

Antimicrobial activity

Antimicrobial activity was assessed for 3 crude and 4 hydrolyzed extracts. All the extracts (20 mg/mL) exhibited an antibacterial activity (inhibition zones Ø= 9 – 15 mm) both against Gram-positive and Gram-negative strains more or less comparable to that of the reference, due to their polyphenols, including lignans content. No antimycotic activity against *C. parapsilosis* was recorded for the 20 µL extract (20 mg/mL) (Table 4). These results could be explained by the eukaryotic structure of pathogenic fungi, which are different from prokaryotic bacteria, thus proving to be more resistant.

When analyzing the antibacterial activity screening of the hydrolyzed extracts, we noticed similar inhibition areas to those obtained for the crude extracts, and comparable to the areas around antibacterial references. The mechanisms responsible for phenolics toxicity upon microorganisms include enzyme inhibition, complexation with extracellular proteins and cell wall disruption.[36,37,38] Our results agree with the fact that natural polyphenols in general, as well as glucosylated lignans (like SDG or SMG), and aglycones in particular (such as SECO or anhydro-SECO) are known to exhibit antibacterial activity.[39,40,41,42]

Thus, both crude and hydrolyzed flaxseed cake extracts look promising as antibacterial ingredients for functional food and cosmetics. However, further studies regarding their interaction with the food and cosmetics matrices are required.

Table 4
Antimicrobial activity of crude and hydrolyzed flaxseed cake extracts

Extracts	Inhibition areas (mm)			
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida parapsilosis*
Crude extract (100% ethanol)	6	10	9	-
Crude extract (80% ethanol)	12	14	15	-
Crude extract (60% ethanol)	12	13	15	-
Hydrolyzed extract (100% ethanol)*	6	ND	9	ND
Hydrolyzed extract (80% ethanol)*	12	ND	15	ND
Hydrolyzed extract (80% ethanol)**	12	ND	15	ND
Hydrolyzed extract (80% ethanol)*	12	ND	15	ND
Gentamicin	17	19	ND	ND
Ofloxacin	21	19	22	ND
Amikacin	ND	21	21	ND
Kanamycin	18	ND	18	ND
Cefuroxime	26	ND	17	ND
Erythromycin	20	10	11	ND
Nystatin	ND	ND	ND	25

*Extract hydrolyzed at 80 °C; **Extract hydrolyzed at 60 °C; ND – not determined; values depicted in the above table represent the mean of three separate assays

CONCLUSION

A very important factor affecting the content of both extracted total polyphenols and lignans is the appropriate solvent polarity, the best results having been obtained with a 60% ethanolic solution. Both crude and hydrolyzed extracts obtained exhibited a remarkable radical scavenging ability, which correlates well with their polyphenolic content ($R^2 = 0.9004$).

As SECO, MATA and LARI were absent in the crude extracts prepared from flaxseed cake analyzed by HPLC, we suggested a preliminary acidic hydrolysis at 80 °C, in order to release the corresponding aglycones successively from the oligomeric chain.

The antioxidant activity of the crude extracts doubled after 2 hours of hydrolysis (2M HCl) at 80 °C, due to the severing of linkages and deglycosylation of phenolic compounds to aglycones, which exhibited an increase in activity. As the DPPH scavenging activities of the flaxseed cake extracts are comparable to other medicinal plant and spice extracts, as well as to those of synthetic antioxidants (BHT, BHA), they could replace conventional additives in food products. Furthermore, flaxseed cake extracts are rich in lignans (phytoestrogens) with anticarcinogenic activity.

The fact that flaxseed cake extracts contain phenolic compounds, including lignans, recommends them as multi-functional antioxidant and antibacterial additives for food products and cosmetics. Obtaining polyphenolic extracts rich in lignans from flaxseed cake represents a sustainable approach because of the high recoverability of this by-product of the cold pressed oil industry. The whole plant of *Linum usitatissimum* could be processed as a renewable resource for textile fibres, edible oils and antioxidant and antibacterial extracts. These could successfully replace synthetic additives in food and cosmetics.

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