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Extraction Method and Evaluation of Phenolics, Flavonoids, Antioxidant Activity, Antimicrobial Activity and Minerals of Bitter *Lupinus albus* in Palestine

Michel Hanania [1]*, Sireen Radwan and Eman Karmi
Department of Chemistry, Bethlehem University, Bethlehem, Palestine

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Abstract: In this paper, extraction methods for bitter *Lupinus albus* seeds are described. Samples were either soaked with water (thereafter soaked) or used without soaking (thereafter unsoaked) and were exposed to Soxhlet extraction using commercial ethanol (~95 %), 80 %, 70 %, 60 % and 50 % ethanol. Yields of extractions were 12.5 %, 23.9 %, 12.6 %, 16.1 % and 16.6 % for soaked samples, and 11.6 %, 20.9 %, 19.3 %, 20.5 % and 25 % for unsoaked samples, respectively. 50 % ethanol extracted samples showed the highest sodium concentration (14.186 $mg.g^{-1}$), 60 % ethanol extracts showed highest potassium concentration (8.76 mg.). Redox titration showed higher concentration of Fe^{+2} in all unsoaked samples. All samples showed very good antimicrobial activity against Gram positive bacteria (*Staphylococcus aureus*) but were weakly active or had no activity against Gram negative bacteria (*Escherichia coli*). FRAP method for the determination of antioxidant activity showed higher antioxidant activity for the soaked samples than the unsoaked ones. Total phenolics showed very similar results for soaked and unsoaked samples. Finally, contents of two of the flavonoids (flavonones and dihydroflavonols) were measured spectrophotometrically against Rutin as standard. Soaked samples demonstrated higher content than those of the unsoaked ones.

Key words: Bitter lupine, antioxidant, phenolics, flavonoids and antimicrobial.

Introduction

Lupinus albus is a yellow small seed that grows on a special tree and is considered a plant that belongs to the *Fabaceae* family. There are two types of lupine: bitter and sweet seeds. Sweet seeds are found in North America while bitter seeds can be found in the Mediterranean region. Studies have shown that lupine is composed of materials and special compounds that carry several health benefits [1-3]. Lupine contains many important components such as: proteins 45 %, carbohydrates 32 % and oils 18-21 %. The seeds are also rich with vitamin K, vitamin C, potassium, manganese and omega 3 and 6 fatty acids [1,4-5]. It is considered a good tonic for the nerves, heart stimulant, diuretic, anti-Kalokzima for some skin diseases and psoriasis, helps reduce sugar in patients and helps relieve constipation and deworming. Lupine is also rich with fibers that provide a suitable nutrient to diabetic patients, given the fact that those fibers slow the body's absorption of glucose resulting from the decomposition of starches and sugars, which resists the occurrence of high sugar level in blood. On the other hand, lupine has some disadvantages that it contains fluoride which is toxic to the human body; for some people it causes nausea and disturbances in the eye and sight [6].

Lupine contains many types of alkaloids, some of which display antimicrobial and antioxidant activities [7-9]. However, alkaloids are responsible for the bitter taste of bitter lupine seeds type and they are neurotoxic and unsuitable for human consumption [10]. The predominant lupine alkaloids are sparteine and lupinine [11].

Human consumption of improperly prepared bit-

*Corresponding author (Michel Hanania)
E-mail: < mhanania@bethlehem.edu >

ter lupine seeds causes plant poisoning in which anticholinergic syndrome is developed due to the ingestion of anticholinergic alkaloids [12]. Only few methods for the isolation of lupine alkaloids are described [13]. Qualitative and quantitative analysis of those compounds is not considered to be an easy task. The percentage of alkaloid in lupine differs from one place to another. So, studying the pharmacological properties of lupine that is consumed in Palestine helps figure out its benefits and the correct use of it in medicine [14]. This research aims at investigating the many benefits and the activity of the extract of bitter lupine seeds.

Experimental part

Bitter lupine seeds were obtained from the local market, while all other fine chemicals were purchased from Sigma Aldrich Company. Deionized water was used in all preparations, while commercial alcohol was used for extraction. Biochrom Libra S22 UV-VIS spectrophotometer was used for the determination of the antioxidant capacity, phenolic content, flavonoids and for the preparation of the McFarland reagent. Model FP 640 flame photometer was used for the measurements of sodium and potassium. Bacteria strains were provided from the Department of Biology at Bethlehem University.

Bitter lupine was either used after being soaked (soaked) or used without soaking (unsoaked). Soaking was performed in water for five days and seeds were then dried, ground and finally used for extraction. Continuous extraction was mainly done utilizing Soxhlet extractor using different ethanol ratios for two and a half hours. Solvent was then evaporated under vacuum and the residue was stored away from direct light.

Determination of sodium and potassium

The concentration of sodium and potassium was determined by flame photometry. A calibration curve was obtained by reading the absorbance of different standard concentrations of potassium and sodium (ppm amounts) at a constant rate. Then the solutions of all extracts were measured and the concentrations of sodium and potassium were calculated from the obtained calibration curves.

Determination of Ferrous ion (Fe^{+2})

Fe^{+2} was determined by redox titration with potassium dichromate in acidic medium. Sodium diphenylamine sulfonate, a pH independent redox indicator, was used which had no color in the reduced form and had a red-violet color when oxidized. When the dichromate reacted with all Fe^{+2} present in the sample, the indicator was oxidized and the endpoint was detected as the violet color formed.

Pharmacological properties

Antioxidant and antimicrobial activities are the pharmacological properties that were studied for soaked and unsoaked lupine.

Antimicrobial activity

Antibacterial activity was studied on lupine against *S. aureus* (Gram positive) and *E. coli* bacteria (Gram negative). Resistance to bacteria was tested by the "well" method. Three wells were created, the first one for negative control; H_2O, the second one for the sample, and the third one for positive control; amoxicillin.

Studies showed that lupine seeds had an antimicrobial activity against standard strains of bacteria. The seeds extract showed significant activity on *S. aureus* while it was weakly active on *E. coli*.

Standard procedures were followed in the preparation of Muller-Hinton agar[22] and McFarland reagent[21], as follows:

Preparation of Muller-Hinton agar [22]

38.0 g of agar was put in 1000 mL deionized water. It was heated to boiling to dissolve the media completely. Then the media was sterilized by autoclaving at 15 lbs. pressure and 120°C for one hour. It was mixed well and poured into sterile petri dishes (20 mL in each) and the agar medium was cooled at room temperature.

McFarland reagent [21]

0.05 mL of 1.175 % barium chloride dihydrate ($BaCl_2.2H_2O$) solution was added to 99.5 mL of 1 % sulfuric acid (H_2SO_4) with continuous stirring to maintain a suspension. The turbidity was verified by using spectrophotometer, the absor-

bance at 625 nm should be (0.008 to 0.1). Barium Sulfate suspension should be vigorously agitated on Vortex mixer before each use.

Amoxicillin

50 mg/100 mL phosphate buffer (pH 6: 2.29 g K_2HPO_4 and 11.8 g KH_2PO_4 / 1000 mL).

Sample preparation

Preparation of samples was done by accurately weighing 500-600 mg of sample and dissolving in 10 mL ethanol and making up the volume to 50 mL by phosphate buffer solution.

Cultivation of bacteria

Two solutions of *E. coli* and *S. aureus* were prepared then bacteria were dispersed by cotton swap on Petri dishes (for soaked and unsoaked lupine sample 50 %, 60 %, 70 % and 80 %). Three wells were created on each petri dish with an appropriate distance between them. 95 µL of each sample was injected to the wells and Petri dishes were incubated at 37°C for 24-36 hours.

The dimensions of the zone of inhibition (ZOI) can be measured as an indicator of the effectiveness of the antibiotic.

Antioxidant capacity

The antioxidant activity or capacity was determined by the ferric reducing antioxidant power (FRAP) [15] method that relies on reduction by antioxidants, of the complex ferric ion-TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine). The binding of Fe^{2+} to the ligand makes a complex that creates blue color intensity. The absorbance can be measured to test the amount of iron reduced and can be correlated with the amount of antioxidants.

Procedures

For the preparation of FRAP reagent, 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3.H_2O$ were prepared and mixed in the ratio of 10:1:1, respectively. FRAP reagent was incubated at 37°C for 10 minutes. Lupine extracts were prepared as (150 mg/100 mL in 60 % ethanol), while $FeSO_4$ (0.1-2 mM) was used for calibration. 80 µL of the extracts was mixed with 1000 µL FRAP and 1000 µL H_2O and the mixture was incubated at 37°C for 30 minutes before using. Absorbance of the colored products was determined at 593 nm against 60 % ethanol as blank.

Total phenolic content

The total amount of phenolic compounds was determined using Folin-Ciocalteu method [16-17], accordingly:

For standard preparation, 1.2 mL of 7.5 % Na_2CO_3 solution was brought with 1.8 mL diluted Folin-Ciocalteu reagent (1:1 in water) and 40 µL of Gallic acid standard (10 - 900 ppm in water). As for samples, 90 µL of the prepared ethanol samples were used instead of the Gallic acid and the mixtures were shaken by Vortex and incubated at 30°C for two hours where color turned to greenish-blue. Absorbance was measured by a proper spectrophotometer at λ = 765 nm using 60 % ethanol as blank.

Flavonoids

The colorimetric identification and quantification of two types of flavonoids (flavonones and dihydroflavonols) is based on their reaction with 2,4-dinitrohenylhydrazine (DNP) in presence of KOH in methanol [18].

Reagents

DNP solution was prepared by dissolving 0.5 g of 2,4-dinitrophenylhydrazine in 1 mL 96 % H_2SO_4 and diluted to 50 mL with MeOH.

KOH/MeOH solution was prepared by dissolving 10 g of KOH in 100 mL 70 % MeOH.

Experimental

Rutin standard (5-100 ppm) was used for calibration. Standards and samples of the final concentration preparation was done by adding into a test tube 0.2 mL of standard or sample and 0.4 mL of DNP solution. Mixture was incubated at 50°C for 90 minutes. After cooling to room temperature 0.8 mL of 10 % KOH was added. From the formed solution, 0.35 mL was placed in a new test tube and diluted to 5 mL total volume with methanol. Finally, the solutions were measured on an appropriate spectrophotometer at λ= 486 nm using pure methanol as blank.

Results and discussion

Extraction of both soaked and unsoaked lupine seeds was performed using ethanol-water system. As Table 1 shows, the highest percentage of extract was obtained using 80 % ethanol in the soaked samples, while 50 % ethanol afforded the best results for unsoaked samples

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Determination of sodium and potassium

The concentrations of sodium and potassium ions were calculated from the calibration curves. Results of sodium and potassium content are illustrated in Table 2. As table 2 shows, unsoaked seeds contained more sodium and potassium than the soaked ones. This might be due to the fact that sodium and potassium containing inorganic compounds were dissolved and removed upon soaking. It is obvious that when the alcohol percentage is increased, the sodium-containing organic compounds are increased. It seems that sodium containing compounds do not dissolve in solvents with lower than 70 % alcohol, while those of potassium need solvents with 60 % alcohol or more for the soaked samples. It was also found that as the alcohol percentages decreased for the unsoaked seeds, the sodium and potassium content is increased with the exception of the 60 % solvent for sodium and the 50 % for potassium.

Determination of iron (Ferrous ion, Fe^{+2})

In determining ferrous ion, unsoaked samples contained higher amounts than the soaked ones, and the best extracting solvent for the soaked samples was the 70 % alcohol, while for the unsoaked was that of 60 %. Results are summarized in Table 3.

Table 1. Percentages of residue obtained from both soaked and unsoaked bitter lupine seeds

Solvent	Soaked	Unsoaked
95 % EtOH	12.5 %	11.4 %
80 % EtOH	23.9 %	20.9 %
70 % EtOH	12.6 %	19.3 %
60 % EtOH	16.1 %	20.5 %
50 % EtOH	16.6 %	24.0 %

Table 2. Sodium and potassium content of extracts (mg/g)

Ethanol percentage	Sodium content (mg/g)		Potassium content (mg/g)	
	Soaked	Unsoaked	Soaked	Unsoaked
95 %	0.6452	0.6478	0.3526	0.9675
80 %	0.2454	8.4505	0.9018	1.1766
70 %	0.2561	10.351	0.5241	-
60 %	0	8.409	0.8559	8.7607
50 %	0	14.186	0	1.1855

Table 3. Concentration of Fe^{+2} in extracts (mg/g)

Ethanol percentage	Soaked	Unsoaked
95 %	1.359	2.931
80 %	1.551	3.540
70 %	2.072	5.278
60 %	1.663	7.025
50 %	1.156	5.264

Antimicrobial activity

Unsoaked lupine extract samples showed good inhibition of gram positive bacteria (*S. aureus*) with good inhibition zones, with the 70 % alcohol extract being the best. Other extracts also gave satisfactory results and are shown in Table 4. Soaked samples showed satisfactory results with the 60 % and 70 % extracts only. Other extracts showed no inhibition (table 4). Our negative antibacterial studies on *E. coli* are in agreement with previous studies [8,19]. Lampart-Szczapa *et al*. found that lupine seeds of bitter lupine species exhibited negative antibacterial activity of the cotyledons against *E. coli* while the testa (coat) of the same seeds showed antibacterial effects [8].

To determine the MIC, serial dilutions of the previous solutions were done and tested. It was found that 40 µg still had minimal effect on the inhibition.

Antioxidant activity

In the antioxidant test, a standard curve was constructed by measuring the absorbance of the ferrous sulfate standard solutions. Samples were measured and the following results were obtained and were expressed as equivalent to (mg) of $FeSO_4$ for each gram of extract (Table 5).

Table 5 reveals that the highest results for both soaked and unsoaked samples were obtained when extracted with commercial alcohol (95 %). This result was expected since antioxidant agents are organic compounds and are more soluble in ethanol. Unsoaked samples contained a little more antioxidants than those soaked samples. It is interesting to learn that the 50 % ethanol extracted results were slightly higher than that of the soaked and unsoaked samples. This might be attributed to the fact that the higher water content was able to dissolve more "inorganic" compounds or salts which have the same activity. These results are compatible with the finding of the ethanolic extract results of a recent study of lupine that showed a marked antioxidant activity [20].

Total phenolic content

Results of total phenolic content for soaked and unsoaked samples are shown in Table 6. Results reveal no big difference between soaked and unsoaked samples owing to the fact that those phenolics are very slightly soluble in water. It is

Table 4. Zones of inhibition for extract against *S.aureus* using Amoxicillin as positive control

Sample	Unsoaked Amount (mg)	Unsoaked Diameter of sample	Diameter of amoxicillin (0.0475mg)	Soaked Amount (mg)	Soaked Diameter of sample	Diameter of amoxicillin (0.0475 mg)
50 %	1.157	8 mm	18 mm	1.022	-	18 mm
60 %	1.155	8 mm	16 mm	1.013	8 mm	14 mm
70 %	1.189	10 mm	14 mm	1.022	8 mm	14 mm
80 %	1.142	6 mm	14 mm	1.026	-	14 mm

Table 5. Antioxidant activity for extracts (expressed as mg $FeSO_4$/g extract)

Extract percentage	Soaked	Unsoaked
95 %	72.16	60.68
80 %	53.78	34.67
70 %	35.92	31.50
60 %	32.56	19.44
50 %	37.50	40.29

worth noting that the 70 % extracts showed the lowest concentration in both samples. These results show a higher phenolic content than the study of Lampart-Szczapa *et. al* [8].

Flavonoids

In this experiment, Rutin was used as standard for the determination of the above-mentioned two flavonoids in samples. Results are summarized in Table 7 for both soaked and unsoaked samples. In both samples the 80 % extracts showed the highest results, while lowest results were obtained with the 60 % extracts. The results are expressed in mg Rutin per one gram of sample.

Summary and Conclusion

Current work explores extraction method of bitter lupine seeds using Soxhlet extractor. It was found that the highest percentage of extract was obtained for the soaked lupine with 80 % ethanol, while best results for the unsoaked seeds were obtained with 50 % ethanol. Lupine was examined to determine sodium, potassium and iron (Fe^{+2}) levels.

The study showed that the concentration of sodium, potassium and iron are higher in soaked seeds, which is related to the fact that sodium, potassium and iron containing compounds are mainly inorganic that were dissolved and removed upon soaking. Lupine showed also good inhibition effect on *S. aureus* (MIC ~ 40 µg), with the 70 % alcohol extract being the best, while no inhibition effect on *E. coli* was observed. It was also found that there were no marked differences in the antioxidant activity and phenolic content before and after soaking, owing to the fact that those antioxidants and phenolics are very slightly soluble in water. Soaked seeds showed higher flavonoids content than the unsoaked, with the 80 % extracts being the highest. Finally, all ethanolic extracts exhibited, to different extent, pharmacological properties and thus, providing a valuable source of nutraceutical supplements. Consequently, lupine can serve as alternative to chemical or synthetic antimicrobials and antioxidants to struggle with spoilage organisms, inhibiting lipid oxidation and thus extending shelf life of some products.

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Table 6. Results of total phenolics (expressed in mg Gallic acid/ g extract)

Ethanol percentage	Soaked	Unsoaked
95 %	47.80	35.56
80 %	45.54	42.33
70 %	36.85	36.70
60 %	45.31	39.57
50 %	42.68	42.18

Table 7. Results obtained for the different flavonoids content

Extraction solvent (ethanol %)	Soaked mg Rutin/1 g sample	Unsoaked mg Rutin /1 g sample
50	23.19	19.21
60	19.55	8.77
70	23.14	23.57
80	32.37	23.55
95	24.77	19.09

References

1. **Shahhat, I., Ghazal, G.M., Mohamed, G.S. (2014).** Effect of Ascorbic acid and Niacin on Protein, Oil Fatty Acids and Antibacterial Activity of Lupinustermis Seeds. International Journal of Pharmacognosy and Phytochemical Research. 6(4): 866-873.
2. **Hassan, E.A., Ibrahim, M.M., Khalifa, Y.A.M. (2012).** Efficiency of Biofertilization on Growth, Yield, Alkaloids Content and Chemical Constitutes of *Lupinus termis* L. Plants. Aust. J. of Basic and Appl. Sci. 6(13): 433-442.
3. **Kattab, H.A. (1986).** Plant wealth in ancient Egypt. Min. Agriculture of Egypt.
4. **Kohajdova, Z., Karovicova, J., Schmidt, S. (2011).** Lupin composition and possible use in bakery a review. Czech J. Food Sci. 29(3): 203-211.
5. **Quiles, M., Oquendo-Jimenez, I., Herreno-Saenz, D., Antoun, M.D. (2010).** Genotoxicity of Alkaloid-Rich Extract from *Lupinus termis* Seeds. Pharm Crops. 1: 18-23.
6. **Australia New Zealand Food Authority, (2001).** Lupin alkaloids in food. A toxicological review and risk assessment. Technical Report Series No.3, pp 1-21.
7. **Wang, S. and Clements, J. (2008).** Antioxidant Activities of Lupin Seeds**.** IN Palta, J.A. and Berger, J.B. (eds). 'Lupins for Health and Wealth' Proceedings of the 12th International Lupin Conference, 14-18 Sept., Fremantle, Western Australia. International Lupin Association, Canterbury, New Zealand. ISBN 086476-153-8.
8. **Lampart-Szczapa, E., Siger, A., Trojanowska, K., Nogala-Kalucka, M., Malecka, M., Pacholek, B**. **(2003).** Chemical composition and antibacterial activities of lupin seeds extracts. Food / Nahrung 47(5):286-290.
9. **Oomah, B.D., Tiger, N., Olson, M., Balasubramanian, P. (2006).** Phenolics and antioxidative activities in narrow-leafed lupins (*Lupinus angustifolius*). Plant Foods Hum. Nutr. 61(2): 91-97.
10. **Kurzbaum, A., Safori, G., Monir, M., Simsolo, C. (2008).** Anticholinergic Syndrome in Response to Lupin Seed Toxicity. Israeli Journal of Emergency Medicine. 8(2): 20-22.
11. **Henry, T. (1913).** The Plant Alkaloids. Reprint. London: Forgotten Books. 410-411.
12. **Williamson, P.M., Highet, A.S., Gams, W., Sivasithamparam, K., Cowling, W.A. (1994).** Diaporthetoxica sp. nov., the cause of lupinosis in sheep. Mycological Research. 98(12): 1364.
13. **Wink, M. (1993).** Methods in Plant Biochemistry. Academic Press Limited. Vol 8 pp 197-239.
14. **Essawi, T. and Srour, M. (2000).** Screening of some Palestinian medicinal plants for antibacterial activity. J. Ethnopharmacology. 70: 343-349.
15. **NurAlam, M., Bristi, N.J., Rafiquzzaman, M. (2013).** Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal. 21: 143-152.
16. **Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M. (1999).** Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology. 299: 152-178.
17. **Marcussi, F., Campos, M.D.C., De Grandis, R.A., Sylos, C.M. (2015)**. Analytical methods applied for the determination of phenolic compounds in lettuce and their antioxidant activity. Academia Journal of Agricultural Research. 3(8): 116-121.
18. **Heim, K.E., Tagliaferro, A.R., Bobilya, D.J. (2002).** Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. J. Nutr. Biochem. 13: 572-84.
19. **Erdemoglu, N., Ozkan, S., Tosun, F. (2007).** Alkaloid profile and antimicrobial activity of *Lupinus angustifolius* L. alkaloid extract. Phytochem Rev 6: 197-201.
20. **Lampart-Szczapa, E., Korczak, J., Nogala-Kaluckaa, M., Zawirska-Wojtasiak, R. (2003).** Antioxidant properties of lupin seed products. Food chemistry. 83: 279-285.
21. **McFarland, J. (1907).** The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. JAMA. XLIX (14): 1176-1178.
22. **Mueller, J.H. and Hinton, J. (1941).** Proc. Soc. Exp. Biol. and Med. 48: 330-333.