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PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE SHELLS OF *ARACHIS HYPOGAEA* L. FRUITS (PEA NUT) CULTIVATED IN EGYPT

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ABSTRACT

β-sitosterol, *β*-sitosterol-α-glucoside, eriodictyol and luteolin were isolated from the aqueous ethanolic extract of pea nut shells after partitioning with petroleum ether, chloroform and ethyl acetate. The identity of the four compounds were done via chromatographic and spectral analysis, as well as comparison with reported data and with authentic samples. *β*-sitosterol-α-glucoside is reported here for the first time from pea nut shells. The amino acids and the protein contents were determined. In addition, the antimicrobial and the anti-inflammatory studies were done.

INTRODUCTION

The pea nut fruits (*Arachis hypogaea* L.) is well known world wide as ground nut. The pea nut is not a true nut but rather a legume of an annual herbs of the Fabaceae. The pea nut is one of the major sources of edible oil and protein meals which considered to be of high valuable nutrition for human and animals. Pea nut being the fifth most important oil seed in the world. The genus comprises 15 species of which only *A. hypogaea* is cultivated[1].

In Egypt about 130-150 thousand fedans[2] are cultivated yearly. The yield ranges from 156-185 million kg every year. The pea nut shells represent 29-32% of this yield, i.e., about 50-60 million kg of useless material are thrown every year.

Previous studies of the pea nut shells cultivated in china U.S.A. reported the isolation of *β*-sitosterol, daucosterol, luteolin, unidentified saponin[3], eriodictyol and 5,7 dihydroxy chromone[4].

In China it had been used in folk medicine for the treatment of hypertension[5].

Reviewing the available literature revealed the lack of information about the constituents of the pea nut shells cultivated in Egypt.

In this study, some of the constituents of light petroleum, chloroform and ethyl acetate fractions of 70% aqueous ethanolic extract were isolated and characterized. Also, the amino acids and the protein contents were determined. The antimicrobial, the analgesic and the anti-inflammatory activities were done.

EXPERIMENTAL

Plant Material:

The shells of the unroasted pea nut fruits were collected in June 2001, by taking off the shells from commercial pea nut from Zagazig.

General Technique :

Column chromatography silica gel 60 (E. Merk), analytical TLC Silica gel GF_{254}, TLC precoated plates (E. Merk), PTLC, precoated silica gel plates (20 × 20 cm), (1mm), solvent system chloroform-methanol (9:1), visualization *p*-anisaldehyde/sulfuric acid spraying reagent and/or U.V. light at 254 nm.

Melting points were determined on a digital melting point apparatus (Electro thermal I.T. 10, England). A Shimadzu U.V. 260 Spectrophotometer was used for the U.V. spectral analysis. MS were measured on a Finnigan SSQ 7000. The amino acids were estimated by using amino acid analyzer Eppendorf LC 3000.

The isolation :

The dried powdered pea nut shells (600 g) was extracted with 70% aqueous ethanol (4L. × 3). The combined extract were evaporated under reduced pressure and the aqueous residue was successively partitioned with petroleum ether (300 ml × 3), chloroform (300 ml × 3) and ethyl acetate (300 ml × 4).

TLC screening of the three extracts, the petroleum ether extract revealed the presence of one major violet spot with R_f 0.86 using chloroform-benzene (1:1) as solvent system. The chloroformic extract showed 2 major violet spots R_f 0.86 and 0.27 after spraying with *p*-anisaldehyde/sulfuric acid and many yellow minor ones using the previous solvent system. While the ethyl acetate extract showed one major yellow spot with R_f 0.35 and two other ones having very close R_f values 0.51 and 0.48 using chloroform-methanol (9:1).

The petroleum ether extract (4 g) was chromatographed over a silica gel column (3 × 80 cm) packed in petroleum ether using increasing amounts of chloroform. Fractions eluted with petroleum ether - chloroform (1:1) gave compound **I** (60 mg).

The chloroformic fraction (4 g) was chromatographed over a silica gel column (3 × 80 cm) packed in benzene using chloroform then gradiently eluted with chloroform then chloroform-methanol. Fractions eluted with benzene-chloroform (3:1) gave compound **I** (about 100 mg) while fractions eluted with chloroform-methanol (9.5:0.5) gave compound **II** (20 mg).

The ethyl acetate fraction (3 g) was chromatographed over a silica gel column (2 × 60 cm) packed in chloroform using ethyl acetate with increasing amounts of methanol. Fractions eluted with chloroform-ethyl acetate (9.5:0.5) contained a mixture of three compounds having R_f 0.51, 0.48 and 0.35 respectively using silica gel plate and chloroform-methanol (9:1) as solvent system.

The mixture, (100 mg) was subjected to PTLC using the solvent system chloroform- methanol (9:1) the band with R_f 0.48 (purple with U.V. light) was eluted with methanol to obtain a whitish solid denoted f_1 (30 mg). The band with R_f 0.35 was eluted with chloroform-ethyl acetate (9:1) to obtain compound f_2 (about 150 mg).

Identification of the Compounds :

Compound I: *β*-sitosterol: white needle crystals, m.p. 140°C. Ms m/z 414 [M$^+$]. The identity was confirmed by comparing the data with those reported for β-sitosterol[5] and COTLC and m.m.p. with authentic sample.

Compound II: β-sitosterol-O-glucoside: White solid, powder m.p. 286°C. Ms, m/z 576 [M$^+$]. It was identified by comparing with reported data[6] and with authentic sample through the COTLC and m.m.p. The aglycone after acid hydrolysis compared with authentic *β*-sitosterol through COTLC and m.m.p. The sugar part was identified as glucose comparing with authentic sample using P.C., butanol – acetic acid – water (4:1:5) as solvent system and aniline phthalate spray reagent which gave brown spot with R_f 0.15[7].

Compound f_1: eriodictyol (5,7,3',4'-tetrahydroxy flavanone), whitish solid powder, (chloroform-methanol) m.p. 264°C U.V.λ max, nm, (MeOH) 286, 324 (sh), + NaOMe 274(sh), 322, 393(sh) + $AlCl_3$: 288, 380, + $AlCl_3$ + HCl : 288, 373, + NaOAC: 287, 322, + NaOAC + H_3BO_3 : 288, 335 (sh).

MS, m/z (rel. abund. %): 288 [M$^+$] (98.5), 287 [M-H]$^+$ (52.9), 271(12), 179(36.8), 152(10.7), 153(100), 136(59), 123(29.4) comparable with reported data[8].

Compound f_2 : luteolin (5,7,3',4'-tetrahydroxy flavone), yellow needles (MeOH), m.p. 328°C U.V.λ max, nm, (MeOH): 254, 350, + NaOMe 270, 408, + $AlCl_3$: 273, 418, + $AlCl_3$ + HCl : 274, 359, 385, + NaOAC: 267, 359, + NaOAC + H_3BO_3 : 260, 373.

MS, m/z (rel. abund. %): 286 [M$^+$] (100), 258 (58), 229(42), 211(13.4), 171(15), 157(19.6), 152(22), 153(69.6), 137(22), 134(78.6), 124(69.6), 123(28.6), comparable with reported data[9].

Analysis of protein contents of the pea nut shells

The determination of protein was as follow: 0.5 g powdered pea nut shells mixed with 10 mls of TCA (30%) and 5 mls of 2% phenol water and left over night then filtered. The filtrate was dried at 50°C followed by the addition of 10 mls NaOH (0.5N) and left for another 24 hrs. at room temperature. The hydrolysate was neutralized using diluted HCl, then the protein contents were determined according to Lowry at al.[10] using bovine albumin as a standard protein.

For the protein amino acids content, 0.5 g of powdered pea nut shells was hydrolyzed with 6N HCl in sealed tube at 120°C for 24 hrs. The hydrolyzed amino acids were estimated by using amino acid analyzer according to black burn method[11]. The results are shown in Table (1).

Table (1): Protein amino acids content of pea nut shell mg/g dry wt.

Aspartat family	Glutamate family	Pyruvate family
Isoleucine 0.044	Histidine (0.165) Proline (5.920)	Alanine (0.840) Valine (0.036) Leucin (0.166)

The protein amino acids content = 7.171 mg/g dry wt.
Protein content = 7.9 mg/g dry wt.

Preliminary antimicrobial activity:

Materials:

All microbial strains used were locally isolated and identified by API-20 system in the Department of Microbiology, Faculty of Pharmacy, Zagazig University.

About 10 mg from the dried aqueous alcoholic extract of pea nut shells were dissolved in 1 ml DMF, to be used in the test. The antimicrobial activity was carried out by cup plate method[12]. The nutrient agar was seeded with the microbial strains of *E. coli*, *Pseudomonas aeruginosa*, and *Proteus sp.* as (Gram-negative bacilli), *Bacillus subtilis* and *Staphylococcus aureus* as (Gram-positive bacilli and cocci) and *Candida albicans* (fungus). By means of cork borer cups were made after agar solidification. Each cup was filled with 100 µl, from the DMF dissolved extract, then the plates were incubated at 37°C for over night. Penicillin (30 µg/ml) and nystatin (100 µg/ml) were used as positive control.

The results were demonstrated as diameters (mm) of inhibition zones and recorded in Table (2).

Table (2): The antimicrobial activity of the pea nut shells extract.

Microorganisms	*Antimic-robial activity*	*Diameter of Inhibition Zones*		
		Extract	*Pencillin*	*Nystatin*
Candida albicans	(++++)	16	–	10
Bacillus Subtilis	(+++)	14	11	–
Staphylococcus aureus	(++)	12	12	–
E. coli	–	–	12	–
Pseudomonas aeruginosa	–	–	12	–
Proteus sp.	–	–	12	–

(++++) highly active, (+++) less active, (++) moderately active , (–) inactive.

The minimal inhibitory concentration (MIC) of the pea nut shells extract was carried out by tube serial dilution technique[12]. The calculated MIC of the extract against *Candida albicans* was 0.19 mg/ml.

Determination of anti-inflammatory activity:

The anti-inflammatory effect of the pea nut shells extract was determined according to the rat hind paw oedema method[13]. The results are shown in figure (1). The results were compared with the hydrocortisone currently used as anti-inflammatory.

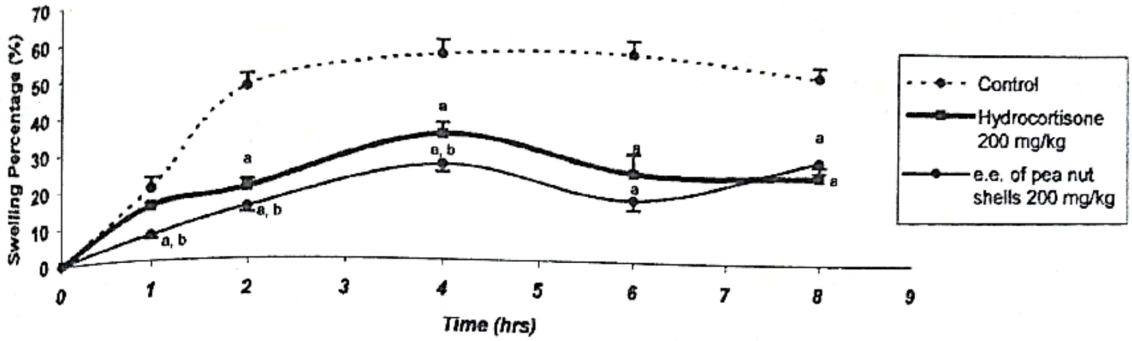

Fig 1. Swelling percentage in the rat hind paw 1,2, 4, 6, 8 hours after injection of 0.05 ml carrageen (1% solution) in normal rats and rats received 200 mg/kg, Hydrocortisone < ir 200 mg/kg ethanolic extract of pea nut shells.
a: Significantly different from control group at P< 0.05.
b: Significantly different from hydrocortisone group at P< 0.05

DISCUSSION

The 70% aqueous ethanol of pea nut shells (A. hypogaea) after solvent fractionation, column chromatography and preparative TLC, affords four compounds. The isolated compounds namely β-sitosterol, β-sitosterol-O-glucoside, eriodictyol and luteolin were identified by comparing their m.p., U.V. data for flavonoids and MS with those reported in the literature[5-8,14,15] on the respective compounds as well as by COTLC and m.m.p.

The protein contents of pea nut shells (7.9 mg/g dry wt.) revealed the presence of considerable amounts of amino acids leucine, isoleucine, histidine and valine which are essential amino acids for humans, alanine and proline as non essential amino acids[16]. This indicate the high nutritive value of this byproduct *Arahis hypogaea* shells.

On the other hand, the antimicrobial activity of the total extract of the pea nut shells showed significant activity against *Candida albicans* and *Bacillus subtilis* which exceed those drugs used as antifungal and antibiotic for Gram positive bacteria.

The anti-inflammatory activity (Fig. 1) of the pea nut shells total aqueous alcoholic extract showed more potent inhibition of the induced oedema than that of hydrocortisoneat 1, 2, 4, 6 and 8 hrs after carrageenin injection, also the onset time of the extract is about 45-60 min, while that of hydrocortisone started its effect after 90-120 min of carrageenin injection. Moreover, the duration of action of the extract was extended for more than 8 hrs after administration having a peak effect reached after 6 hrs, which is shared with hydrocortisone. This effect is due to the presence of considerable amounts of β-sitosterol which has a potent anti-inflammatory and antipyretic effect as reported by **Gupta et al**[17]. The total extract contains also considerable amounts of flavonoids mainly luteolin and eriodictyol as well as other minor compounds which may enhance this activity.

Finally, from all these studies it becomes clear that the pea nut shells have great value as food as it contains considerable amount of protein and also as antifungal, antimicrobial for Gram +ve bacteria and with potent anti-inflammatory activity.

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دراسة المحتويات الكيميائية والتأثيرات البيولوجية لقشر ثمرة الفول السوداني الذي يزرع في مصر

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تم في هذا البحث استخلاص قشر ثمرة الفول السوداني الجافة الغير محمصة بواسطة خليط من الكحول والماء بنسبة ٧٠% كحول ثم تم تجزئة الخلاصة بعد تركيزها الى خلاصة مائية وإلى خلاصة الإثير البترولي وخلاصة الكلوروفورم وخلاصة خلات الإثيل.

تم فصل البيتا سيتوستيرول من خلاصة الإثير البترولي والكلوروفورم وجلوكوزيد البيتا سيتوستيرول من خلاصة الكلوروفورم كما تم فصل مادتين فلافونيتين هما ايريودكتيول والليتيولين من خلاصة خلات الإثيل.

وقد تم التعرف على التركيب الكيميائي لهذه المركبات عن طريق إجراء التحاليل الطبيعية المختلفة مثل التحليل الطيفي بالأشعة فوق البنفسجية وطيف الكتلة وتعيين درجة الانصهار كما تم مقارنتها بعينات مرجعية .

كذلك تم تحديد نسبة الأحماض الأمينية والبروتين لهذه القشرة بالاضافة الى ذلك تم دراسة تأثير هذه الخلاصة الكحولية على الميكروبات وثبت فاعليتها وتأثيرها القوى على الميكروبات الموجبة الجرام والفطريات ، وكذلك تم دراسة تأثير هذه الخلاصة كمضادة للإلتهابات مقارنة بالهيدروكورتيزون وأثبتت أن لها فاعلية كبيرة.