Antimicrobial proteins and oil seeds from pumpkin (*Cucurbita moschata)*.

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Abstract: The nutritive value and biological activity of pumpkin (*Cucurbita moschata*) seeds cultivated in Egypt were evaluated. Chemical analysis of fiber, protein, ash, carbohydrates, and fatty acids present in the non irradiated and irradiated seeds was conducted. The results show that the values for the indices are within recommended levels for edible oils. Seeds were found to be rich in oil (44.45±2.83 %). The oil contains an appreciable amount of unsaturated fatty acids (71.10±4.32 %) and found to be a rich source of linoleic acid (52.64±0.90 %).Gamma irradiation of pumpkin increased significantly ($P<0.05$) the yield of free fatty acid, acid value and peroxide value of extracts. Results showed decreases in the iodine value after irradiation at doses up to 10kGy. The antimicrobial effect of irradiated and unirradiated pumpkin oil seeds was studied. Gamma radiation up to 10kGys don't affect on the antimicrobial activity of pumpkin oil. Three different proteins were extracted from the pumpkin rinds, seeds, and pulp. All the extracted proteins were screened for their antimicrobial activity against the tested microbial isolates. The total protein and antimicrobial effect of all extractions were decreased at gamma irradiation doses used.
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1. Introduction

The pumpkin is an angiosperm belonging to the cucurbitaceae family. *Cucurbita moschata* is more tolerant to harsh environmental conditions than other cucurbitaceae species [1]. Pumpkin fruit has many nutritional components including pumpkin polysaccharides, active proteins, essential amino acids, carotenoids, and minerals. It has been received considerable attentions in recent years because of the nutritional and health protective value of these components [2].

Pumpkin seeds have a high nutritional value, provides good quality oil, and excellent source of protein [3]. In addition to good health benefits, pumpkin seeds are less expensive and are widely distributed. In the traditional medicine in North America and Mexico, pumpkin seeds have been used as an anthelmintic agent and for supportive treatment in functional disorders of the bladder [3].

The healing powers of plants have been used for hundreds of years; about 80% of the available therapeutic substances are originated from medicinal plants [4, 5]. Scientists showed that the plants had medicinal properties for their biological activities ranging from antimicrobial to antitumor. The antimicrobial activity of plants has many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [6, 7].

While some of the oils used on the basis of their reputed antimicrobial properties have well documented *in vitro* activity [8]. The seed of pumpkin has pharmacological activities such as anti-diabetic [1], antifungal, antibacterial and antiinflammation activities, and antioxidant effects [9]. The most critical health benefit attributed to pumpkin seed oil is preventing the growth and reducing the size of the prostate [10].

In this study, the pumpkin (*Cucurbita moschata*) was screened for antimicrobial compounds from seeds oil extracts and all of its under-utilized products (rinds, seeds and pulp).

Recently increased attention has been focused on the utilization of under-utilized agricultural products, as well as by-products and wastes from food processing to produce food and feed. Such utilization would help maximize available resources and minimize waste disposal problems.

Pumpkin seeds, rinds and pulp, that remain in large quantities as waste product after the removal of the flesh could be utilized. The seeds of pumpkin are rich in oil and protein and the crop could potentially become another source of vegetable oil and protein.

The aim of the present study was to investigate the detailed proximate chemical composition, the physicochemical properties of oil and the antimicrobial effect of the extracted oil and proteins. The effect of gamma irradiation on the physicochemical properties and antimicrobial effect of pumpkin oil and extracted proteins were studied.

Sylvia, 2004). These microbes benefit for the plant through different mechanisms action, including the production of secondary metabolities, antibiotics and hormone like substances (Ozbay and Newman 2004; Harman *et al.* 1996). The production of siderophores, antagonistic to soil borne root

pathogens (Dubeikovsky *et al.* 1993; Siddiqui *et al.* 2008) has been also reported.

The bio-efficiency of compost therefore, could also be further enhanced by fortifying it with plant nutrients or biocontrol inoculants such as *Trichoderma* spp. *Trichoderma harzianum* alone or in combination with compost has been documented as the most common and effective biocontrol agent for disease control in various host-pathogen systems (Elad 2000; Ibrahim 2005; Siddiqui *et al.* 2008).

Therefore, this study was carried out to determine the efficiency of compost fortified with *T. harzianum* as an alternative to chemical fungicide and Top.Znformulation on morpho-physical growth and occurrence of root rot disease of orange and mandarin citrus seedlings. The effect of different treatments on rhizosphere soil microflora was also studied.

2. Material and Methods:

Experimental procedure

Sample collection and irradiation

Pumpkin *(Cucurbita moschata)* fruits were purchased from Agricultural Research Center (ARC) in Giza (Egypt). The ripe pumpkins were cut and the seeds were extracted from them, washed, dried in an oven at 60 – 70°C to constant weight. Samples of pumpkin seeds were sealed in polyethylene bags and irradiated to 1, 3, 6, and 10 kGy using a Cobat-60 gamma chamber 4000A. INDIA, the average dose rate of this gamma radiation source was 2.5 kGy/h. The irradiation facilities were located at the National Center for Radiation Research and Technology, NCRRT (Nasr City, Cairo, Egypt).

Oil extraction

Control (Non-irradiated seeds) and γ-irradiated pumpkin seeds were ground in an electric grinder to pass through 0.4 mm screen and fed into a soxhlet extractor with methanol as a solvent. The solvent was then distilled off under vacuum at 45 °C in a rotary evaporator [11].

Proximate composition

Moisture content was determined according to AOAC, 1995 [12]. Crude oil was determined by a Soxhlet extractor using methanol as a solvent. Crude proteins were calculated from the nitrogen content by Kjeldahl method (AOAC, 1995) [12]. Crude fiber was determined according to the gravimetric procedure of AOAC, 1995 [12]. Ash was determined by incinerating at 550°C in a muffle furnace for 6 hrs [12]. The total carbohydrate content (on dry weight basis) was determined by subtracting the sum of the percentages of moisture, crude fat, fiber, protein and ash from 100 [100-(crude protein + crude lipids + ash + crude fiber)]. All the analyses were done in triplicate.

Oil quality attributes

Physicochemical properties of oil extracted from irradiated and non-irradiated pumpkin seeds were determined. Peroxide value (mg/kg), iodine value (g/g), free fatty acid (%) and acid value (mgKOH/g) were estimated according to AOAC, 1995 [12].

Fatty acids composition of pumpkin seed oil.

Individual fatty acids were determined by gas liquid chromatography (GLC) [13].

Microorganisms, inoculum and sample preparation

Microorganisms

The bacterial and fungal strains used in this study were obtained from the Microbiology Laboratory, Department of Microbiology, and National Center for Radiation Research and Technology, NCRRT.

Seeds oil (extracted from non-irradiated seeds and γ-irradiated seeds) were screened for their antimicrobial activity using broth micro dilution method. The yeasts species tested were *Candida albicans* and *Rhodotorula rubra*, the mold species tested were *Trichoderma viride*, *Penicillium chrysogenum*, *Rhizopus oligosporus, Aspergillus fumigatus*, *Aspergillus parasiticus*, *Aspergillus niger*

The bacterial species tested were two gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and three gram-negative bacteria (*Klebsiella pneumonia, Pseudomonas aeruginosa,* and *Escherichia coli*)

All the microorganisms used were checked for purity and maintained at 4°C in slants of nutrient agar and sabouraud dextrose agar (SDA) for bacteria and fungi, respectively.

Preparation of inocula

A loopful of cells were transferred from new slant into 25 ml broth medium in 250-ml Erlenmeyer flasks and cultivated on a rotary shaker at 200 rpm for 18 hrs at 37 °C for bacteria, and at 48 hrs for yeasts. Inocula were prepared by transferring morphologically similar colonies of each organism into (5 ml) 0.9 % sterile saline solution to obtain the required working suspensions, 10^8 cfu/ml for bacteria and 10^7cfu/ml for yeasts [14]. For the fungi, fungal spores were harvested after 7 days old SDA slant culture was washed with 10ml saline in 2% Tween 80 with the aid of glass beads to help in dispersing the spores. The spores' suspensions were standardized to 10^5cfu/ml.

Preparation of plates

Stock solutions of the seeds oil and the positive control drugs ampicillin for bacteria and ciprofloxacin for fungi (Sigma-Adrich, UK) were prepared in dimethyl sulphoxide (DMSO) at the concentrations of 100 mg/mL and 1.6 mg/mL,

respectively. Microdilution susceptibility testing was performed in flat-bottom 96-well clear plates containing broth medium (0.1 ml) in each well. Sample solutions (0.1 ml) were subsequently serially diluted two-fold in the plates with the broth, starting with the final concentration of 1000 mg/L for plant extracts and 8 mg/L for standard antibiotics. The working inoculum suspension (0.1 ml) was added. Sterility and growth controls in the presence of organic solvents employed in sample preparation were also included. No inhibitory effects were observed in the presence of DMSO at the highest concentration used (0.5% v/v). The plates were incubated at 37 °C for 24 - 48 hrs for bacteria, and at 30 °C for 5-7 days for yeasts and fungi, respectively. The amount of growth in the wells containing the agent was compared visually with the growth in the growth control wells. The concentration with a prominent decrease in turbidity was determined as the MIC. Each isolate was tested in three separate replicates. Sample with the final concentration of 1000 mg/L for plant seeds oil extracts were not effective with some tested microorganisms so concentrations of 2000 mg/L and 3000 mg/L of plant extracts were also tested for them.

Extraction and irradiation of crude protein from different parts of pumpkin

One hundred grams of dried pumpkin rinds, seeds and pulp were homogenized in extraction buffer (50 mM Tris/HCl, pH 6.8 glycerol 10%w/v, ascorbic acid 0.1%, cyctiene hydrochloride 0.1w/v) using laboratory blender (Stomacher 400). The homogenate was centrifuged and soluble extracts were separately extracted in cold acetone for 30 min. After centrifugation all the extracts were concentrated under vacuum and stored at 4 °C. The filtrates were used as crude extracts. All the procedures were performed at about 4 °C.

Extracted pumpkin protein from seeds, pulp and rinds samples were irradiated at 1, 3, 6 and 10 kGy using a Co^{60} gamma-irradiator at the National Center for Radiation Research and Technology, Atomic Energy Authority, Nasr City, Cairo, Egypt. The dose rate was 2.5 kGy/h. The unirradiated and irradiated collected protein fractions were subjected to an antifungal and antibacterial assay against the tested isolates.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was run in a gel containing 15% (w/v) polyacrylamide and 0.1% (w/v) SDS [15]. The silver staining method for protein was used, as a sensitive and detective stain of little protein (2µg) in a single band [16]. The total protein content for each extracted protein was determined [17]. The proteins gels were scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different M.W. of bands were determined against PCR marker amresco 100 bp K180 by unweighted pair-group method based on arithmetic mean (UPGMA)

Antimicrobial Growth Inhibition Assay

Antibacterial activity assay of extracted proteins.

In vitro antibacterial activity of extracted proteins was determined by the agar disc diffusion method. 18 ml of sterilized Mueller Hinton agar medium was taken in each Petri dish and then spread with a suspension of the tested micro-organism (average concentration is 10^8 cells/ml). 150 µg aliquots of each extract was applied on sterile paper discs and placed on the seeded agar plates and then incubated at 37°C for 24 hrs. The antibacterial activity of the test agent was determined by measuring the mean diameter of zone of inhibitions in millimeter [18].

Antifungal activity assay

Fungal fragments were placed in the center of potato/ dextrose/agar (PDA) plates, and the plates were then incubated at 28°C for 60 hrs. After incubation, sterilized blank paper disks were placed around the seeded fungi and 150 µg aliquots of each protein extract was then delivered to the disks. The plates were incubated at 28°C for 72 hrs [19].

Analysis of uptake with acridine orange.

To determine whether extracted proteins induce permeabilization of the microbial cell membrane, we assayed the uptake of the vital dye, acridine orange, by conidia and vegetative cells of yeasts and bacteria. Microbial cells non treated with extracted proteins were used as a control [20].

Statistical Analyses

The statistical package used was SAS system version 9.1.3 (Cary, NC). Each determination was carried out on three samples. Averages and least significant differences were calculated. A P value of <0.05 was considered significant.

3. Results and Discussion

Proximate chemical composition.

The proximate chemical composition of dried pumpkin seeds is shown in Table 1. The contents of moisture, protein, lipid, ash, fiber and Carbohydrate were: 7.73 ± 1.22, 39.25 ± 0.51, 44.45 ± 2.83, 4.41±0.32, 3.60 ± 0.45 and 8.52 ± 0.98%, respectively. However, most of those components were in accordance with those reported for *Cucurbita moschata* seeds by many investigators (21-23).

The whole seed moisture contents was quite low (7.73 ± 1.22%) and was within the range of moisture The pumpkin seeds are a good source of protein (39.25 ± 0.51 %), oil (44.45%) and carbohydrates (8.52 ± 0.98%). Similar values for

protein and oil contents of the pumpkin seeds were reported [22].The crude protein value compared favorably with high protein seeds and legumes like soybeans (35%) and cowpea (22.7%); however, it is higher than others such as lima beans (19.8%) and chickpeas (19%) [23].

The total ash content of the seeds in the present study (4.41±0.1) is similar to those obtained by another study [21] which ranged from 3.5-5.3%. These values are similar to those of soybean (5.0%), cotton seed (4%), sesame (3.8% and sunflower seed (4.1%) [23].

The carbohydrate content (8.52 ± 0.98%) was lower than that reported, meanwhile, the crude fiber content of kernel was similar to that reported [23].

The high oil and protein content makes the seed a potential source of commercial vegetable oil and protein.

Effect of Gamma Irradiation on Chemical Constituents:

The effect of gamma irradiation (1, 3, 6, and 10 kGy) on the proximate chemical composition for both non-irradiated and irradiated pumpkin seeds are presented in table 1. There are no appreciable differences in proximate composition of samples irradiated to 1, 3 and 6 kGy (Table 1). The moisture content was not substantially affected by gamma irradiation. Similar findings showed that gamma irradiation has no real effect on moisture content of oil seeds [24]. Protein, fiber, and ash content were not significantly ($P<0.05$) changed. This result is in agreement with other study, which reported that gamma irradiation did not induce any change in protein, fiber, and ash content of groundnut [25]. Similarly another study reported that the protein and crude fiber contents of almonds did not change after irradiation to 3, 7 and 10 kGy [26]. No significantly ($P<0.05$) differences were observed in carbohydrates content and in the total lipids was observed with 10 kGy radiation dose. No significant differences between irradiated and unirradiated walnuts in moisture, ash and protein contents were found [27]. Data obtained by other authors also showed that gamma irradiation, using doses up to 10 kGy, did not induce significant loss in water soluble components such as sugars and proteins [28]. The oil content of non-irradiated and irradiated seeds with 10 kGy was 44.45% and 42.80%, respectively. It was also found that there is an inverse relationship between oil content and irradiation dose, namely, if irradiation dose increases, oil content decreases [29].

Table (1): Effects of different doses of gamma irradiation on proximate chemical composition of pumpkin seeds.

*Nutritive Content (g/100g d.w.)	Control	Radiation Dose			
		1 kGy	3 kGy	6 kGy	10 kGy
**Moisture	7.73^{a} ± 1.22	7.37^{a} ±1.20	7.68^{a} ±0.08	7.63^{a} ±0.24	7.20^{a} ±0.37
Protein	39.25^{a} ± 0.51	39.09^{a} ±0.74	39.09^{a} ±0.50	39.25^{a} ±0.50	39.00^{a} ±1.10
Lipid	44.45^{a} ± 2.83	44.45^{a} ±2.83	44.40^{a} ± .20	43.63^{a} ±3.10	42.80^{a} ±2 .05
Ash	4.41^{a} ± 0.32	4.41^{a} ± 0.65	4.41^{a} ± 0.60	4.38^{a} ± 0.60	4.34^{a} ± 0.62
Crude fiber	3.60^{a} ± 0.45	3.60^{a} ± 0.50	3.60^{a} ± 0.40	3.55^{a} ± 0.85	3.50^{a} ± 0.35
Carbohydrate	8.52^{a} ± 0.98	8.41^{a} ±1.13	8.46^{a} ±1.07	9.20^{a} ±0.33	10.22^{a} ±0.47

*Each value in the table is the mean of three replicates (n=3), ±SEM. SEM: standard error of the mean

$^{A, b, c}$ Means with different superscripts in the same row are statistically different ($P<0.05$) according to Least Significant Test (LSD). **Moisture content (g/100g f.w.)

Physicochemical properties

The physicochemical properties of the pumpkin seed oil (control) were determined. It was found that, acid value (mg KOH/g of oil), peroxide value (equiv.g O_2/Kg of oil) iodine value (g /100g of oil) and free fatty (%) of the oil were 4.54±1.42, 0.85±0.58, 105.53±12.29, and 2.27±0.42, respectively.

The oil extracted from the dried pumpkin seeds was liquid at room temperature. Acid value is an indicator for edibility of oil and suitability for industrial use. Pumpkin seeds oil has 4.54±1.42 mgKOH/g while, this falls within the recommended codex of 10 mgKOH/g for edible oils [30]. The results suggest that the oils are edible and can also be used in the manufactured of paints and vanishes as comparable to the values of [31].

The iodine value which is useful in predicting the drying properties of oils is 105.53 (g /100g of oil).The

high iodine value of this oil indicates that they have a high content of unsaturated fatty acids suggests that the oil may be used for cooking purpose [30]. The iodine value is also an index for assessing the ability of oil to go rancid [32]. The high iodine values indicate that the oils have longer time to undergo oxidative deterioration [33].

The peroxide value was low (0.85 ± 0.46 meq peroxide /kg), as the Codex Alimentarius Commission stipulated permitted maximum peroxide levels of 10 meq peroxide/kg oil for soybean, cottonseed, rapeseed, and coconut oils. [30] These values were comparable to those reported for pumpkin seed oil [23, 34, and 35).The peroxide index is an indication of the amount of hydroperoxides present in oil. These compounds arise from lipid oxidation. High peroxide values are associated with higher rate of rancidity. The low peroxide values of the oils indicate that they are less liable to oxidative rancidity at room temperature [28, 29]. This shows the commercial potential of the oil, which is enhanced by the low peroxide value, free fatty acids, and acid values.

Effect of Gamma Irradiation on Oil Quality Attributes:

Two cases were studied, firstly pumpkin dry seeds were exposed to gamma radiation (0, 1, 3, 6, and 10 kGy) and oil extracted from these irradiated seeds. The second case was the irradiation of oil which extracted from non irradiated seeds with the same gamma radiation doses as the first case.

The effect of gamma irradiation (0.0, 3, 6 and 10 kGy) on iodine value, acid value, peroxide value, and free fatty acids for both of seeds and oils are illustrated in table 2. The results indicate that, tested doses of gamma radiation have no significant ($P<0.05$) effect on the seeds. The highest iodine values were acquired from the oil of the sample which is not exposed to irradiation; and relating to the irradiation dosages this values decreased significantly ($P<0.05$), and finally the lowest values recorded in the sample treated with 10 kGy irradiation. However, the changes were higher for acid and peroxide values, the changes in irradiated samples were higher than that of non-irradiated, and the changes in irradiated oil were higher than irradiated seeds. Irradiated oil was more sensitive (1kGy) to gamma irradiation than irradiated seeds. The effects of gamma irradiation on irradiated oil appear with the radiation dose 3 kGy and above. Similar findings were reported [36] for the iodine value of sunflower and soybean oil which decreased significantly with high gamma radiation (1, 5 and 20 KGy) while the acid values were increased. The decrease in iodine value may be attributed to the saturation of the double of unsaturated fatty acids bonds [27].

Our results are consistent with other study, which also reported a decrease in the iodine value upon irradiation [37]. Radiation probably broke some double bonds and induced oxidation processes in the fatty acids resulted in saturation [29]. These results also agree with other studies, which found that, the unirradiated samples have highest iodine values, suggesting saturation of oils as a result of irradiation [27, 38].

It was found that, prior to irradiation, pumpkin seeds and seeds oil exhibited very low peroxide values, indicative of good product quality in terms of degree of lipid oxidation. PV increased with an increase in irradiation dose. Maximum peroxide value was observed in pumpkin oil irradiated at 10kGy. Present results are in general agreement with those obtained for soybean oil [28], walnuts oil [39] and almonds oil [40].

Peroxide value characterizes quantity of peroxides formed in the oils as intermediates of oxidative reactions under irradiation and at high temperatures [41]. Previously, an increase in the peroxide value was attributed to interaction of gamma radiation with fat molecules, which triggered oxidation, dehydration and polymerization reactions [42]. A previous study of cashews irradiated to 7 kGy revealed approximately a five-fold increase in the peroxide value [43].

The increase in free fatty acids of the oil might be due to slight and random hydrolysis of triglycerol molecules to free fatty acids and diacylglycerols [29, 27].

Fatty acid composition

The fatty acids composition of pumpkin seeds oil is presented in Table3. The main monounsaturated fatty acid (MUFA) present in non-irradiated seeds was oleic acid (17.2±0.63), with minute levels of Erucic (0.71±0.13%) and palmitoleic acid (0.15±0.06) present. The major polyunsaturated fatty acid (PUFA) present was linoleic acid (52.64±0.90) with small amounts of linolenic acid (0.40±0.32). The major saturated fatty acids present were palmitic acid (19.01±0.86) and stearic acid (9.5±1.23) with small amounts of myristic acid (0.39±0.05).

Our results were in agreement with other studies which, observed that, linoleic acid was the principal fatty acid followed by oleic acid in pumpkin seed oil [21, 44]. This result indicates that the fatty acid composition of pumpkin seed oil is quite close to that of melon seed oil [45]. The presence of high amounts of the essential linoleic acid suggests that the pumpkin seed oil is highly nutritious.

Table (2): Effect of different doses of gamma irradiation on physico-chemical parameters (properties) of oil quality of irradiated pumpkin oil.

parameters	Irradiation case	Radiation Dose (kGy)				
		0	1	3	6	10
Acid value (mg KOH/g of oil	Seeds	4.54^{a} ±1.42	5.54^{a} ±1.02	6.06^{a} ±0.80	6.15^{a} ±0.72	6.67^{a} ±0.69
	Oil	4.54^{c}±1.42	7.24^{bc}±0.42	8.16^{abc}±0.72	9.86^{ab}±0.52	12.7^{a} ±0.50
Peroxide value (equiv.g O_2/Kg of oil)	Seeds	0.85^{a} ±0.58	0.88^{a} ±0.50	1.10^{a} ±0.34	1.30^{a} ±0.28	2.25^{a} ±0.63
	Oil	0.85 ^{b}±0.58	1.53^{b}±0. 26	2.63^{b} ±0.36	4.80^{b}±0.40	42.5^{a} ±3.22
Iodine value (g /100g of oil)	Seeds	105.53 ^{a} ±12.2	101.96 ^{a} ±10.2	97.76^{a} ±11.69	94.39^{a} ±9.85	87.25^{a} ±9.3
	Oil	105.53 ^{a} ±12.2	84.43^{ab}±8.13	76.56^{ab} ±7.40	72.80^{ab} ±8.20	63.46^{b}±7.37
Free Fatty acid (Oleic acid %)	Seeds	2.27^{a} ±0.42	2.77^{a} ±0.57	3.03^{a} ±0.38	3.07^{a} ±0.25	3.33^{a} ±0.56
	Oil	2.27^{c}±0.42	3.62^{bc}±0.64	4.08^{bc} ±0.30	4.93^{ab} ±0.72	6.35^{a} ±0.62

*Each value in the table is the mean of three replicates (n=3), ±SEM.
SEM: standard error of the mean
$^{A, b, c}$ Means with different superscripts in the same row are statistically different (P<0.05) according to Least Significant Test (LSD).

Table (3): Fatty acid composition % of *Cucurbita moschata* seeds

Fatty acid (FA)	Mean value
Myristic (C14:0)	0.39±0.05
Stearic (C18:0)	9.5±1.23
Palmitic (C16:0)	19.01±0.86
Erucic (C22:1)	0.71±0.13
Palmitoleic (C16:1)	0.15±0.06
Oleic (C18:1)	17.2±0.63
Linoleic (C18:2)	52.64±0.90
Linolenic (C18:3)	0.40±0.32
Total saturated FA	28.90±2.14
Total Monounsaturated FA	18.06±0.82
Total Polyunsaturated FA	53.04±1.22
Total unsaturated FA	71.1±2.04
R1=% TSFA/% TUSFA	0.406

The changes of fatty acid content of irradiated pumpkin oil and oil extracted from irradiated seeds are given in Table 4. At low irradiation doses, small changes were observed in saturated and unsaturated fatty acids components, and the changes in fatty acids composition of oil extracted from irradiated seeds were not significant (P<0.05).

It was observed that, for irradiated oil, parallel to the increases in irradiation doses, there is an significant (P<0.05) increase in total saturated fatty acid (SFA) from 28.90 % to 54.29 %, i.e., there is a decrease in total unsaturated fatty acid (TUSFA) from71.10% to 45.71% for irradiated oil, respectively (Table 4). Another study suggested that, the decrease in unsaturated fatty acids during the irradiation exposure of oil is mainly due to a molecular structure change in fatty acids [38].

It is apparent that higher the unsaturation of fatty acids the higher is their oxidation potential. Thus, the increase in SFA concentration may be explained by the oxidation of PUFA and MUFA, respectively. The ratio of total unsaturated over total saturated acids (PMUFA+PPUFA/PSFA) was used [46] to predict the shelf life of hazel nuts; indicating that the lower the ratio, the longer was product shelf life. In the present study, this ratio was 0.406 prior to irradiation increasing to 0.474 and1. 188 after irradiation of seeds and oil, respectively.

The high level of unSFA in these oils was due to their high levels of linoleic acid. This showed that these oils are good sources of unSFA, mostly PUFA, with linoleic acid (an essential fatty acid) being the most abundant (52.64%). Linoleic acid is the most important essential fatty acid, for it must be got from food [47].

Some reported no significant changes in poly-unsaturated fatty acids of almond kernels irradiated up to a dose of 7 kGy, while monounsaturated fatty acids decreased with a respective increase in saturated fatty acids [40]. They also added that, the highly unsaturated fatty acids were very sensitive and readily destroyed by irradiation. Generally, most saturated components are increased as a function of irradiation dose whereas unsaturated components decreased with increasing total dose [40].

Antimicrobial activity of pumpkin seeds oil.

Methanolic extracts of pumpkin seeds (crude oil) were exposed to gamma-rays radiation at dose levels of 1, 3, 6, and 10 kGy. The effects of different doses of gamma irradiation on the antimicrobial activity were studied. The non-irradiated methanol extract of the oil used as control.

Table (4): Fatty acid composition % of non-irradiated seeds and irradiated pumpkin oil

	Fatty acids	Radiation doses (kGy)				
		0	1	3	6	10
Seeds	**Monounsaturated**	18.06^{a} ±1.42	17.61^{a} ±2.58	17.51^{a} ±1.25	17.46^{a} ±2.29	17.06^{a} ±2.78
	Polyunsaturated	53.04^{a} ±2.36	52.81^{a} ±0.95	52.76^{a} ±2.78	52.77^{a} ±3.42	50.74^{a} ±2.69
	Total unsaturated	71.1^{a} ±3.25	70.42^{a} ±1.87	70.27^{a} ±4.13	70.23^{a} ±3.25	67.80^{a} ±2.63
	Total saturated	28.90^{a} ±1.75	29.58^{a} ±0.40	29.73^{a} ±1.29	29.77^{a} ±4.02	32.20^{a} ±2.41
	R1=% SFA/%USFA	0.406	0.420	0.423	0.424	0.474
Oil	**Monounsaturated**	18.06^{a} ±1.42	17.55^{a} ±2.58	16.78^{a} ±1.25	15.37^{a} ±2.29	14.69^{a} ±2.78
	Polyunsaturated	53.04^{a} ±2.36	50.45^{ab}±2.10	39.47^{bc} ±1.34	35.20^{bc} ±3.55	31.02^{c}±1.22
	Total unsaturated	71.1^{a} ±3.25	68.0^{ab} ±3.10	56.25^{bc} ±2.06	50.57^{c} ±1.42	45.71^{c}±1.55
	Total saturated	28.90^{c} ±1.75	32.0^{bc} ±1.47	43.75^{ab} ±2.33	49.43^{a} ±3.54	54.29^{a} ±2.65
	R1=% SFA/%USFA	0.406	0.470	0.778	0.978	1.188

*Each value in the table is the mean of three replicates (n=3), ±SEM. SEM: standard error of the mean
$^{A, b, c}$ Means with different superscripts in the same row are statistically different (P<0.05) according to Least Significant Test (LSD).

Antibacterial activity

In the present study, two-fold serial dilution technique was used to determine the minimal inhibitory concentration (MIC) of crude methanolic extracts against the selected bacterial strains. The results revealed (Table5) that the methanolic seeds extracts of un-irradiated (control) samples have antibacterial effects against *Bacillus subtilis*, *Staphylococcus aureus* , *Escherichia coli* and *Klebsiella pneumonia* local isolates at concentration levels of 1.0, 2.0, 2.0 and 3.0 mg/ml, respectively. The plant extracts were found ineffective against *Pseudomonas aeruginosa* at concentration levels up to 3000 µg/mL. The results regarding the effect of gamma irradiation on antibacterial activities showed that there was no difference in the activity of the irradiated extracts up to a dose level of 10 kGy. No information in the literature is available on the effect of gamma irradiation on the antibacterial activity of pumpkin oil seeds. Little is also known for other plant materials.

Previous studied [48] showed that gamma-radiation treatment did not have any detrimental affect on the antimicrobial activity of the *Nigella sativa* seed up to 10 kGy radiation doses. Similarly, the microbial decontamination of tea by gamma irradiation was studied [49] and the results showed that, the antimicrobial and sensory properties of the samples were unaffected by radiation treatment within a dose of 10 kGy. Another study [50] on ciprofloxacin showed that the antimicrobial activity was not affected by gamma-irradiation treatment up to 100 kGy treatment.

Antifungal activity

The methanolic extracts of irradiated and unirradiated (control) pumpkin oil seeds samples were checked for antifungal activity using two-fold series dilution method. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2µl, from the micro plate wells without visible growth, into micro plates containing 100µl of broth per well and further incubation for 72hrs at 28°C. The lowest concentration with no visible growth was defined as the MFC, indicating=99.5% killing of the original inoculum.

The results indicated that the unirradiated oil have antifungal activity against *Rhodotorula rubra* and *Candida albicans* at 0.5 and 1.0 mg/ml concentrations, respectively and *Rhodotorula rubra* was the most susceptible isolate to the seeds oil. The effective concentration of pumpkin oil seeds methanolic extracts were 1.0 mg/ml against *Penicillium chrysogenum* and *Aspergillus parasiticus* and 2.0 mg/ml against *Aspergillus flavus (A)* , respectively.

No antifungal activity was detected against other selected fungi. Following exposures to gamma rays, the irradiated samples of the plant did not show any change in antifungal activity. This revealed that the antifungal activities of plant extracts against selected fungi were not affected by gamma irradiation up to 10 kGy doses.

This is in agreement with a previous study [48] on *Nigella sativa* seeds, which showed that gamma irradiation did not affect the antifungal activity.

Table (5): Effect of different doses of gamma irradiation on the antimicrobial activity of pumpkin seeds oil.

Microorganism (strain)	Irradiation dose (kGy)				
	0	1.0	3.0	6.0	10
Pseudomonas aeruginosa	>3.0	>3.0	>3.0	>3.0	>3.0
Klebsiella pneumoniae	3.0	3.0	3.0	3.0	3.0
Escherichia coli	2.0	2.0	2.0	2.0	2.0
Staphylococcus aureus	2.0	2.0	2.0	2.0	2.0
Bacillus subtilis	1.0	1.0	1.0	1.0	1.0
Candida albicans	1.0	1.0	1.0	1.0	1.0
Rhodotorula rubra	0.5	0.5	0.5	0.5	0.5
Aspergillus niger	>3.0	>3.0	>3.0	>3.0	>3.0
Aspergillus flavus(A)	2.0	2.0	2.0	2.0	2.0
Trichoderma viride	>3.0	>3.0	>3.0	>3.0	>3.0
Aspergillus flavus(H)	>3.0	>3.0	>3.0	>3.0	>3.0
Penicillium chrysogenum	1.0	1.0	1.0	1.0	1.0
Rhizopus sp	>3.0	>3.0	>3.0	>3.0	>3.0
Aspergillus fumigates	>3.0	>3.0	>3.0	>3.0	>3.0
Aspergillus parasiticus	1.0	1.0	1.0	1.0	1.0

*MIC values expressed in (mg/ml)
*Maximum concentration tested was 3 mg/ml
*>3.0=No inhibitory effect up to the concentration level of 3000 µg/ml Experiments run in triplicate.

Antimicrobial activity of different crude extracted proteins from pumpkin.

This study determined the inhibitory activity of different extracted crude proteins, (extracted from seeds, rinds and pulp) for growth of different microorganisms, including bacteria (Gram +ve and Gram -ve), yeasts and fungi. The average of the diameters of the growth inhibition zones obtained on the experiments is shown in Table 6.

Extracted pumpkin seeds crude protein had a higher antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis*, the inhibition growth zone diameter were 10.0 and 8.0 mm, respectively.

The study revealed that the extracted rinds crude protein had a higher antimicrobial activity of (27.5, 25.0, and 23.0 mm) against *Penicillium chrysogenum*, *Aspergillus flavus(A)*, and *Aspergillus flavus(A)*, respectively.

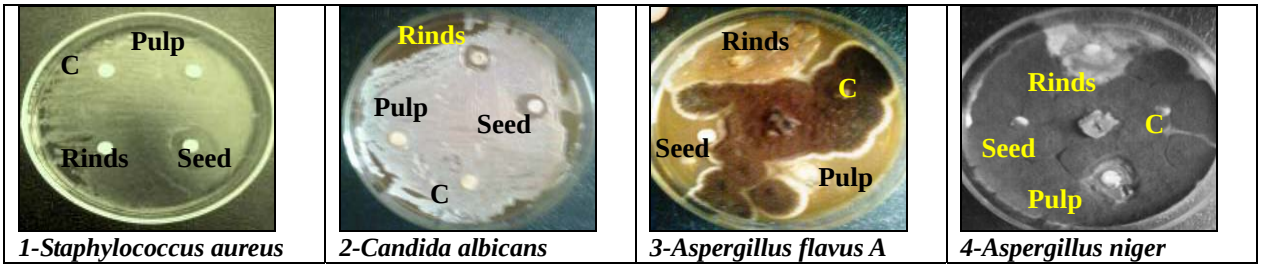
It is clear from the results (Table 6) that the seeds protein was effective against tested Gram +ve bacteria and had no effect against Gram –ve bacteria.

Pulp protein was effective on all tested bacteria, on the other hand, the rinds protein had no effect against all tested bacteria and *Aspergillus parasiticus* by the tested concentrations used in this study.

In another study, Gram-negative bacteria have been reported to be more resistant than Gram-positive to proteins and oils antimicrobial effect because of their cell wall lipopolysaccharides which may prevent these active compounds reach the cytoplasmic membrane of gram-negative bacteria [51].

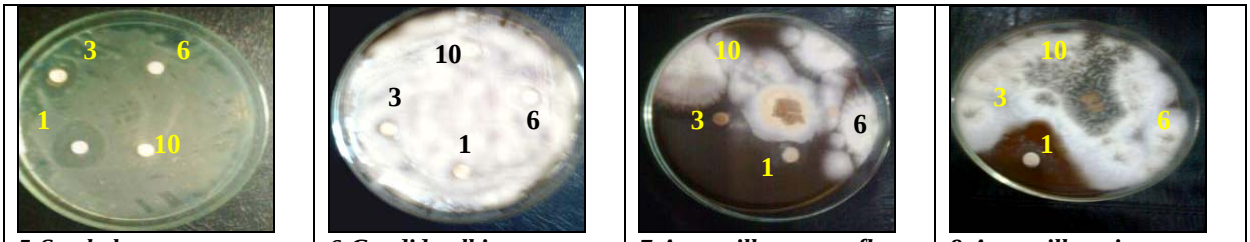
The study evaluated the *in vitro* effect of all extracted proteins on the growth of the tested fungi and bacteria after gamma irradiation (1, 3, 6 and 10 kGy). Antimicrobial effect of the extracted proteins was found to be decreased with the increased of the irradiation doses used. Another study attributed this decrease in the antimicrobial effect of the extracted proteins to the changes in protein fractions which may be related to some cross linking or aggregation of proteins as a result of gamma irradiation which could affect in protein nitrogen [52].

The antimicrobial activities of the extracted proteins and the effects of gamma radiation on it were visible on the microbial growth inhibition zones as shown in (Figs 1, 2).



1-Staphylococcus aureus | *2-Candida albicans* | *3-Aspergillus flavus A* | *4-Aspergillus niger*

Fig (1): Inhibitory activity of extracted proteins for microbial growth.



5-Staphylococcus aureus **(pulp protein)** | *6-Candida albicans* **(Seed protein)** | *7-Aspergillus flavus A* **(Rinds protein)** | *8-Aspergillus niger* **(Rinds protein)**

Fig (2): Effect of different doses of gamma radiation (1, 3, 6, 10kGy) on the inhibitory activity of extracted proteins for microbial growth.

Table (6): The antimicrobial activity of extracted crude proteins from different parts of pumpkin

Microorganisms	Extracted crude proteins		
	Seeds	Rinds	Pulp
Pseudomonas aeruginosa	-	-	2.0
Klebsiella pneumoniae	-	-	1.0
Escherichia coli	-	-	1.5
Staphylococcus aureus	10.0	-	12.0
Bacillus subtilis	8.0	-	9.0
Candida albicans	5.0	4.0	-
Rhodotorula rubra	9.0	6.0	8.0
Aspergillus niger	-	5.0	4.5
Aspergillus flavus(A)	10.0	25.0	4.0
Trichoderma viride	-	4.0	-
Aspergillus flavus(H)	-	4.0	-
Penicillium chrysogenum	10.0	27.5	-
Rhizopus sp	1.0	2.0	1.0
Aspergillus fumigates	-	23.0	-
Aspergillus parasiticus	-	-	-

*(150 µg /disc) *Diameter of inhibition in millimeters (mm) *- No inhibition with the tested concentration

The final preparations gave single bands on a 15% SDS-PAGE gel. The molecular mass of the purified proteins were determined to be 56, 59, and 30 kDa for rinds, pulp, and seeds, respectively by 15% SDS-PAGE gel Fig (3). The computer analyses of protein spots on SDS-PAGE gels were carried out. The results in Table (7) showed that 21 specific different proteins were detected in rinds and pulp, respectively and 23 specific different proteins were detected in seeds. Their molecular weights (M_w) were ranged from 183 to 28 kDa. The computer analysis of the proteins showed 91.19 % similarity between the rinds proteins and pulp proteins and there were 84.40% similarity between them and seeds proteins.

Antimicrobial proteins from pumpkin have been previously identified. Three basic proteins

(MAP2, MAP4 and MAP11) from the pumpkin seed inhibited the growth of yeasts [53]. Cucurmoschin, an antifungal peptide isolated from black pumpkin seeds inhibited growth of *Botrytis cinerea, Fusarium oxysporum* and *Mycosphaerella oxysporum* [9]. A ribosome-inactivating protein derived from *Cucurbita moschata* had antibacterial activity against phytopathogenic bacteria *Phytophora infestans*, *Erwinia amylovora* and *Pseudomonas solanacearum* [54]. In addition, PR-5, with a molecular mass of 28 kDa and high homology to thaumatin, was isolated from pumpkin leaves that exhibited a synergistic effect with nikkomycin, a chitin synthase inhibitor, against *Candida albicans* [55].

PR-5 proteins have been isolated from *A. thaliana* [56], corn [57], beans [58], and many other plants [59, 60, and 61]. The majority of PR-5 proteins have molecular masses of; 22 kDa and are stabilized by eight disulfide bonds. This highly stabilized structure allows PR-5 proteins to be very resistant to protease degradation [62].

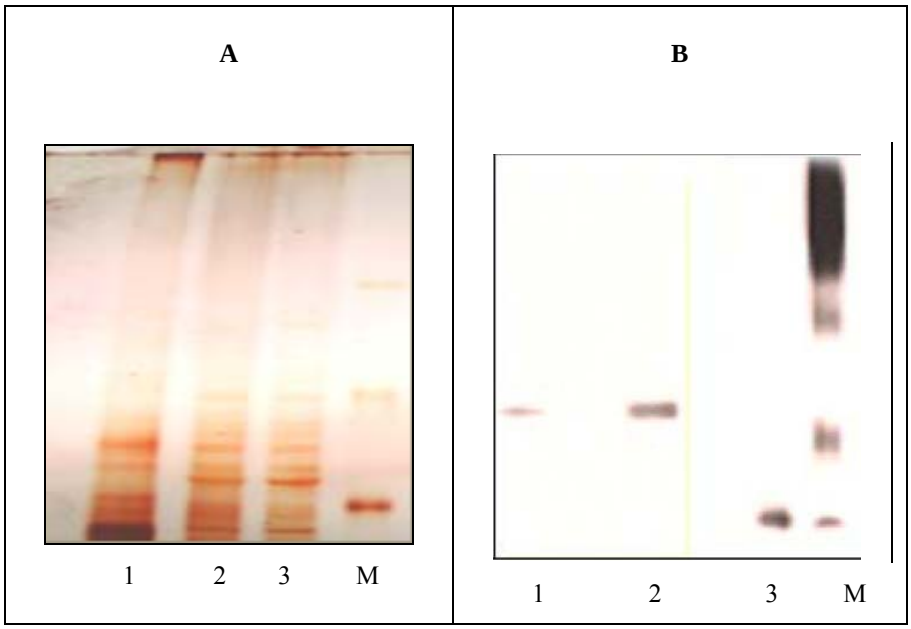


Fig 3: SDS–PAGE of crude (A) and purified (B) proteins analyzed by SDS-PAGE on a 15% separating gel with silver nitrate. A-crude proteins extract from pumpkin rinds (lane 1), crude proteins extract from pumpkin pulp (lane 2), crude proteins extract from pumpkin seeds (lane 3), molecular size marker (lane M). B- Purified protein extract from pumpkin rinds with M_w 56 (kDa) (lane 1), purified protein extract from pumpkin pulp with M_{w59} (kDa) (lane 2), and purified protein extract from pumpkin seeds with $M_{w\,30}$ (kDa) (lane 3). Positions of the marker (M) proteins (116, 66 and 45 kDa) were represented on the right hand side of gel.

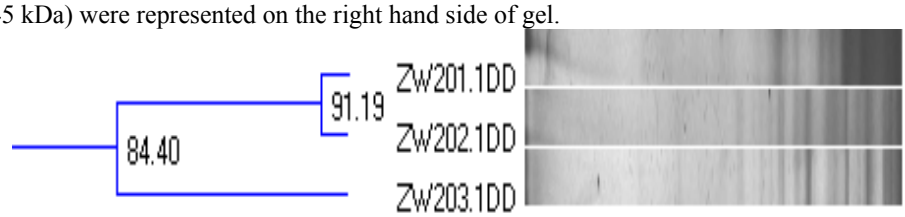


Fig 4. Similarity between the extracted proteins.
*1-rinds proteins 2- pulp proteins and 3- seeds proteins.

Table (7): Molecular weight and percentage (%) of extracted proteins from Different parts of pumpkin.

Peak number	Rinds proteins		Pulp proteins		Seeds proteins	
	M_w (kDa)	%	M_w (kDa)	%	M_w (kDa)	%
1	183	4.95	183	2.43	183	3.29
2	170	11.60	177	6.62	177	8.36
3	115	3.42	119	0.86	119	2.46
4	103	2.32	111	3.52	98	5.72
5	97	1.26	99	5.02	80	7.47
6	89	5.24	77	4.59	76	3.60
7	75	5.98	66	4.10	66	4.95
8	72	1.38	63	1.02	65	2.03
9	69	1.24	61	4.13	62	1.72
10	63	2.00	59	22.83	60	2.40
11	61	4.62	56	4.31	58	2.12
12	56	15.07	52	3.27	56	1.94
13	54	0.49	50	5.33	55	4.75
14	52	4.09	45	2.45	52	3.05
15	46	5.04	43	6.99	49	6.05
16	44	3.15	40	3.23	45	1.57
17	40	6.89	37	3.66	43	5.60
18	39	1.66	35	5.77	40	3.35
19	36	8.66	32	1.34	37	3.24
20	34	3.07	30	5.37	35	6.10
21	28	7.87	27	3.15	31	1.08
22	-	-	-	-	30	15.61
23	-	-	-	-	27	3.54

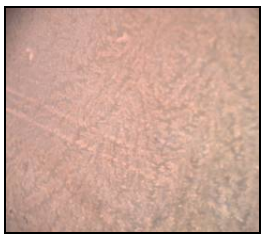
- M_w= Molecular weight

Although the precise mechanism of action of PR-5 proteins is not completely understood, there are a number of interesting observations that may eventually lead to a unified hypothesis for how these proteins function to kill fungi [60, 61]. Several antifungal proteins cause cell permeability changes in fungal cells with a cell wall but have no or little effect on protoplasts [62]. Uptake of the vital stain, SYTOX Green, was enhanced when fungal conidia were treated with Pr-1 suggesting that the protein has membrane permeabilization activity. The author suggested that, Pr-1 induces the damage of the plasma membrane of fungal cell directly, with resultant leakage of cytoplasmic components to the exterior of cell. [63].

The effect of the extracted protein on the cell wall proteins of the tested microorganisms were studied by staining the fungal spores and vegetative cells of bacteria and yeasts by acridine orange. It is clear (Fig 5) that the tested proteins may be having lyses or destruction effect on the sporangia cell wall and the cells of yeasts and bacteria.

Acridine orange, the first stained dye in yeast, turns into green fluorescence in viable cells and orange fluorescence in dead cells. Acridine orange used for the detection of contamination in beer and

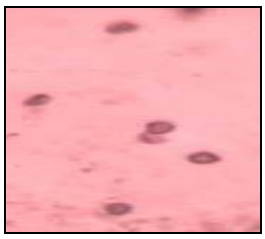
food as it has the ability to differentiate viable and dead cell distinction [64].



1-Candida albicans **(Control)**

2-Candida albicans **(Seed protein)**

3-Aspergillus flavus **(Control)**



4-Aspergillus flavus **(Rinds protein)**

Fig (5): Effect of extracted proteins on the viability of microbial cells. Live cells (1, 3). Uptake (2, 4) of acridine orange by dead cells (orange stained).

4. Conclusion and Recommendations:

This study showed that the Egyptian pumpkin seeds had high content of protein and oil indicating high nutritive value. The high content of pumpkin seeds oil indicates that this oil can be extracted and refined for uses.

These oils are very rich in essential fatty acids (linoleic acid). The acceptable acid and peroxide values, high linoleic and low linolenic acid levels of these oils suggest that they could be sources of good edible oils. The abundance of linoleic followed by oleic acid in these oils makes them good oils for reducing serum cholesterol and LDL and increasing HDL levels, hence could be good oils for the fight against cardiovascular illnesses.

The extracted crude pumpkin oil and proteins (seeds, rinds, and pulp) were examined for antimicrobial activities, before and after gamma irradiation. Gamma radiation at dose level above 3 kGy affect on the physico-chemical properties of the pumpkin oil. No changes in the antibacterial activity against tested pathogens after radiation treatment up to10 kGy. In conclusion, the present study indicates that radiation at higher doses may be not good for certain biological activities and may cause degradation or changes in chemical structures of some biologically active important ingredients. This investigation suggests that radiation treatment up to 3 kGy is safe and beneficial for pumpkin seeds.

These findings also indicate that the extracted oil and proteins from pumpkin may be of importance to clinical microbiology and have therapeutic applications.

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