Egypt. J. Chem. **Vol. 66**, No. 10 pp. 133 - 143 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Exploitation of Wheat Germ for the Production of Wheat Germ Oil and Microbial Pufas and Their Potential Application as Wound Healing Agents for Human Skin Fibroblast Cell Line

Mohamed U. Nooman[a], HishamA. Abd El-lateaf[b], Abd El-Nasser A.Khattab [c] Mona M. Rashad [a], Amr S. Al-kashef [a]*

[a]*Biochemistry Department, National Research Centre, Cairo, 12622, Dokki, Egypt*
[b]*Oils and fats Department, National Research Centre, Cairo, 12622, Dokki, Egypt*
[c]*Genetics and Cytology Department, National Research Centre, Dokki, Giza 12622, Egypt*

Abstract

Agro-industrial wastes have environmental negative effects leading to climate changes if improperly managed. This study aimed to utilization of wheat germ as one of the most generated agro-industrial wastes to produce two valuable products and investigation of their potential application as wound healing agents. Wheat germ oil (WGO) was firstly obtained by cold pressing for the wheat germ, then poly unsaturated fatty acids (PUFAs) were produced through microbial conversion of remaining wheat germ oil cake using locally isolated *Saccharomyces cerevisiae.* Fatty acids composition of the extracted WGO and produced PUFAs were assessed by GC/MS. Finally, their impact as wound healing agents against Human Skin Fibroblast cell line (HSF) and antimicrobial effect on two common wound infectious bacteria were carried out. The extracted WGO was 14.6% (w/w) from the wheat germ while, the yield of produced PUFAs was 10.13 g/100 g substrate. Chemical analysis of the wheat germ oil cakerevealed its high contents of protein and carbohydrates (38.57 and 39.17%, respectively). However, fatty acids composition showed that both WGO and PUFAs contained 64.07 and 61.43 % poly unsaturated fatty acids, respectively. Results indicated that PUFAs were more efficient that WGO in the ability to enhance wound healing of HSF at the first 24h. and the growth inhibition of both *Pseudomonas aeruginosa* and *Staphylococcus aureus* by 15 and 25%, respectively.

Keywords: Wheat germ; PUFAs; Microbial conversion; Oil Cake; Wound healing; Human Skin Fibroblast.

1. Introduction

Polyunsaturated fatty acids (PUFAs) are compounds responsible for the regulation of biological functions, as they are a structural component of every living cell membrane. Furthermore, they are precursors to eicosanoids that are hormone-like chemicals affecting the immunological, circulatory, and neurological systems, such as prostaglandins, thromboxanes, and leukotrienes. PUFAs are also found in the immunocompetent cells such as neutrophils and monocytes. Generally, PUFAs are not generated in sufficient amount in the human body, therefore they must be included in food supply. The avoidance of PUFAs depletion is necessary for life, due to the negative consequences of PUFAs deficiency on the kidney, heart and skin along with the nervous, reproductive, respiratory, endocrine and immune systems (Aita et al., 2018 & Ferreira et al., 2021).

On the other side, PUFAs occur in the animal and plant kingdom, such as marine fish and vegetable oils. Other sources of PUFAs are obtained through algae, fungi and bacteria. The PUFAs production from agricultural and animal sources are generally insufficient due to season, climate and geographical location. However, microbial production of PUFAs is the appropriate alternative, as it is not related to climatic and geographical conditions, in addition to its economic aspects because microorganisms actually, may be utilized to grow on agro-industrial

*Corresponding author e-mail:**amralkashef@gmail.com**; (**Amr S. Al-kashef**).
EJCHEM use only: Received date 04 December 2022; revised date 11 January 2023; accepted date 24 January 2023
DOI: 10.21608/EJCHEM.2023.178673.7265

residues as substrates. Realistically, such residues represent an alternative substrate for the growth of microorganisms due to their high content of nutrients (Sarker et al., 2015; Aita et al., 2018 and Al-kashef et al, 2018). Another reason that my encourage the PUFAs microbial production, is the presence of abundant agro-industrial wastes worldwide, that can be used as renewable and sustainable substrates (Nooman et al, 2017).

As evidence for such availability, oil extraction of different seeds in oil industries, actually produce large amounts of agro-industrial wastes known as oil cakes, on the other hand, the oil crop production has expanded by 240 % globally in the last 30 years, while area and yield have increased by 82 and 48%, respectively. The processed oil seeds, produce wide range of oil cakes such as olive, sunflower, safflower, sesame, mustard, soy bean and palm kernel. (El-Hamidi and Zaher 2018; Ancuța and Sonia 2020). Generally, they may be used in animal feeds due to their high protein content (15 to 50 %) or discarded in the environment because of their antinutritional and toxic compounds leading to serious environmental threats (Sharma et al., 2013).

Another example for the processed oil seeds, wheat germ which is a valuable by-product obtained from the wheat milling industry that accounts about 25 million tons/year worldwide. Wheat germ is mainly used for animal feed due to its high nutritional contents namely lipids (10–15 %), proteins (26–35 %) and 17 % carbohydrates. However, its oil has a significant industrial value in food, medicine and cosmetics (Brandolini et al., 2012; Marti et al., 2014; Li et al., 2016 & Gili et al., 2018). Wheat germ oil cake is the remained waste after oil production from wheat germ, with a broad but not yet fully utilized potential for different applications due to its high enzymatic activity. It is also, has a nutritional value rich in amino acids, residual fatty acids, carbohydrates, minerals, vitamins and tocopherols, which in turn, makes it a suitable environment for the growth of micro-organisms (Çalışkan and Özçıra 2019; Arslan et al., 2020 & Starzyńska-Janiszewska et al., 2021). Therefore, the utilization of such waste for the microbial production of PUFA compounds considered to be a reasonable solution for less production cost and waste recycling for a clean environment (Nooman et al, 2017).

PUFAs however, have anti-inflammation, antioxidation, restorative actions and have defense effect against numerous pathogens, including multidrug-resistant bacteria. This in fact, may allow PUFAs to play an important role in the re-regulation of biological functions such as contribution in the wound healing process. Wounds are usually a result of many causes such as natural physical injury, surgery, or burns. Generally, this process includes an overlapping three stages that can be summarize in the inflammation occurs after the injury then proliferation, and remodeling (Silva et al., 2018; Ummalyma et al., 2019 & Kotronoulas et al., 2020). Usually, wounds heal in a period of 7 to 10 days and any delay in this process can extend the tissue damage leading to the chronic wound due to the pathogenic bacterial invasion (Edwards and Harding, 2004; Menke et al., 2007).

Depending on the previously mentioned, wheat germ as a residual of wheat milling industry was utilized in the present study to obtain wheat germ oil (WGO), then another valuable product namely PUFAs were economically produced by the microbial conversion of the remained oil cake using the locally isolated yeast strain (Saccharomyces cerevisiae) in addition to, studying the chemical composition of WGO & PUFAs. To the best of our knowledge, the effect of microbial origin PUFAs on wound healing has not been studied therefore, this study aimed also at the investigation of potential application of the produced PUFAs compared to wheat germ oil (WGO) as wound healing agents in vitro and their anti-microbial effect against two common bacterial wound pathogens.

2. Material and methods

2.1. Substrates

Wheat germ was obtained from Elwatania milling, El Sadat city, Menoufia, Egypt, as by-product of the flour milling process. While, castor oil cake was obtained from Unit of Pressing and Extracting Natural Oils, NRC, Cairo, Egypt. Crude soybean oil was obtained from the Food Technology Research Institute, Soy Processing Centre, Agriculture Research Centre, Giza, Egypt. The Staphylococcus aureus NRRL B-767, used in the antimicrobial evaluation was kindly obtained from Microbiology and Immunology Dept., Faculty of Medicine, Al-Azhar University, Cairo, Egypt. While, Pseudomonas aeruginosa ATCC 9027 was kindly presented by Genetics and Cytology Department, Biotechnology Research Institute, National Research Centre, Cairo, Egypt.

2.2. Extraction of WGO

Wheat germ was pressed with laboratory-type Carver hydraulic press under 10.000 Ib in-2 pressure for 1 hr at room temperature according to (Üstun et al., 1990), then the obtained WGO was immediately kept in -20 ˚C until used. The oil cakes after cold press were air-dried for 24 hrs at room temperature and then ground with commercial blender to a fine

powder, packed in polyethylene bags and stored in deep freezer at -20 ˚C.

2.3. Chemical analyses of the oil cakes

The chemical constituents of the castor and wheat germ oil cakes were assessed by evaluating of their protein, carbohydrate, fat, moisture and ash contents according to A.O.A.C. (1980).

2.4. Yeast strains

2.4.1. Yeast strains isolation and separation

The yeast strain was isolated from castor oil cake whereas, the strains isolation was performed by suspending 1 gm of oil cake sample, in 9 ml sterile distilled water. The mixture was severely vortexed and serially diluted up to 10-3 by sterile distilled water. Then, from each dilution 0.1 ml spread by swab on the malt extract agar medium contains 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar, which was used to select the yeast strains. After the incubation periods of 24 and 48 hrs, the culture fluid was spread by swab on the cetyltrimethylammonium bromide (CTAB)-agar medium, at final 5.5 pH and the fast growth colonies were isolated.

2.4.2. DNA extraction

Genomic DNA was extracted from pure yeast strain (W5-2) culture, that has been isolated from castor oil cake, after cultivated on malt extract using i-genomic BYF DNA extraction Mini Kit (iNtRON Biotechnology Inc., South Korea) following the manufacturer´s instructions (Sambrook et al., 1989).

2.4.3. PCR partial amplification and sequencing of internal transcribed spacer (ITS)

The identification of the yeast strain (W5-2), was carried out based on molecular genetic analysis utilizing the initials ITS. PCR partial amplification and sequencing of internal transcribed spacer (ITS). The partial sequences of the isolate 18S rDNA were received using a protocol depending on Boekhout et al. (1994) method. The amplification of divergent domain of the gene was carried out utilizing different primers: the first one (ITS1) sequence: 5' TCCGTAGGTGAACCTGCGG-3'; while the second primer (ITS4) sequence: 5' TCCTCCGCTTATTGATATGC-3'. The primers were obtained from Operon Technologies, Inc., (Netherlands). The utilized primer (12 ng) in addition to the purified DNA sample (40 ng) were added to each polymerase chain reaction (PCR) bead. Then, the total volume of the amplification reaction was completed to 25 µl with distilled water. The amplification protocol was performed as follows: denaturation for 5 min. at 95°C (each of the 35 cycles comprised of the following segments: denaturation at 95°C for 1 min; primer annealing at 55°C for 2 min and DNA polymerization was carried out by incubation at 72°C for 2 min). At the end, the PCR was stored at 4 °C for analysis. Using a 1.0% agarose gel and 1X TBE (Tris-borate-EDTA) buffer, the amplified DNA product was electrophoresed at a continuous 100 V for approximately 2 hours. The assessment of different band sizes were carried out against 100bp DNA Ladder H3 RTU (GeneDireX Inc., Taiwan) then, bands were stained with ethidium bromide (0.5 µg/ml). Finally, the bands were imaged by Gel Documentation System with UV Trans eliminator.

2.4.4. Yeast DNA purification and identification

The PCR product was purified by Gene JET™ PCR Purification Kit (Termo K0701). The DNA sequencing of the purified PCR product was achieved using ABI 3730xl DNA sequencer (GATC Company, Germany) by utilizing forward primer.

2.4.5. Phylogenetic analysis

The obtained DNA sequence of the isolated yeast was then compared with the sequences available by the Basic Local Alignment Search Tool (BLAST) in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). The sequences were aligned together with the reference taxa covered by the public databases. The evolutionary distance was generated based on parameter model (Jukes and Cantor 1969) and phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987).

2.5. Production of PUFAs

2.5.1. Inoculum preparation

A loopful of the stock culture (7 days old) of the isolated and identified yeast (Saccharomyces cerevisiae) was inoculated in 50 ml sterile inoculum substrate (Wickerham, 1951), that was then incubated in Orbital Shaker, Thermo SCIENTIFIC- USA. at 28 °C and 180 rpm for 24hrs.

2.5.2. Cultivation conditions

A solid-state fermentation (SSF), medium was implemented with some modifications on the method of Rashad et al., (2014a) as follows: 5 g of wheat germ oil cake and 1.5 g of soybean oil were mixed with 4 mL of nutrients solution consisting of ($g \bullet L^{-1}$) NH_4NO_3, 1.0; K_2HPO_4, 2.55; NaH_2PO_4, 0.15; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.1; $MnSO_4.H_2O$, 0.02; peptone, 1.0. Final pH was adjusted to 8, and one ml of the overnight culture (1x 108 cell ml^{-1}) was mixed for seeding thoroughly to the above sterilized

media (121 °C, 30 min) for 8 days and incubated in static condition at 28–30 °C.

2.5.3. Extraction of PUFAs

The extraction was carried out for PUFAs, using a 100 ml of ethyl acetate for each culture flask, then shaken at 160 strokes min−1 for 60 min at 40 °C with an Orbital Shaker, Thermo SCIENTIFIC- USA. After that, each flask had additional volume (25 ml) of hexane for another 30 min then shaken at 160 strokes at 40 °C with the shaker. Through Whatman No.1 the extract was then filtered. Finally, the mixture of extracted sample with solvents was evaporated by rotary evaporator (heidolph Hei-VAP Advantage G1, Germany) at 35 °C to obtain the PUFA sample.

2.6. Fatty acid composition of the WGO and PUFAs

Qualitative analysis of fatty acids composition was estimated by GC/MS technique (MassHunter GC/MS, SW version: Acquisition B.07.03.2129 18-May-2015, Agilent Technologies, Inc.) at the Central Laboratory, National Research Centre, Egypt.

2.7. Cytotoxicity assessment of the produced PUFAs

The cytotoxicity of the produced PUFAs was estimated at Nawah Scientific Inc., Cairo, Egypt. The experiment was approved by the Medical Research Ethics Committee of the National Research Centre (NRC, Cairo, Egypt) (approval no. 20-111) (November 2020).

2.7.1. Cell culture

Human Skin Fibroblast cell line (HSF) was preserved in Dulbecco's Modified Eagle Medium (DMEM), supplemented with streptomycin (100 mg/mL), 100 penicillin (units/mL) and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

2.7.2. Cytotoxicity experiment

The HSF cell viability was estimated calorimetrically by Sulforhodamine B (SRB) assay. A suspension of 100 pL cell (5x103 cells) in 96-well plates incubated for 24 hrs in complete media. Cells were treated with 100 pL media containing PUFAs at different concentrations. After exposure for 72 h of tested PUFAs, the cells fixation was carried out by replacing media with 150 pL of 10% TCA then, incubated for 1 hr at 4 °C. The TCA solution was eliminated, and the cells were washed by distilled water (5 times). Then incubation of cells with 70 pL SRB solution (0.4% w/v) in a dark place at room temperature for 10 min was performed. Plates were washed using 1% acetic acid (3 times) then air-dried overnight. After that, of 150 pL of TRIS (10 mM) have been added in order to dissolve the protein-bound SRB stain. Finally, absorbance was measured at 540 nm utilizing a BMG LABTECH®- FLUO star Omega microplate reader (Ortenberg, Germany). The half maximal inhibitory concentrations (IC50) were then reported.

2.7.3. Wound healing assay

The WGO and produced PUFAs were investigated as wound healing aid as follows: the HSF cells were plated (2x105/well) onto a coated 12-well plate for wound scratch assay and cultured in 5% Fetal Bovine Serum- Dulbecco's Modified Eagle Medium (FBS-DMEM) then incubated at 37 °C in 5% CO2. Next day, horizontal scratches were introduced into the cells monolayer; the plate was well washed by phosphate buffer saline (PBS), control wells were renewed with fresh medium while tested wells were treated with fresh media containing examined samples. Images were taken periodically using an inverted microscope for 72hrs during the incubation time. The images were analyzed by MII Image View software version 3.7 (Skehan et al., 1990; Rodriguez et al., 2005; Jonkman et al., 2014; Rueden et al., 2017; Main et al., 2019; Martinotti & Ranzato, 2019).

2.8. Anti-microbial experiment

10 µL of sample, subsequently diluted to 100 µL with phosphate buffer saline (PBS; NaCL 8 g L^{-1}, KCL 0.2 g L^{-1}, Na_2HPO_4 1.44 g L^{-1}, KH_2PO_4 0.24 g L^{-1}, Tween 80 1mL L^{-1}, pH 7.4). The samples (1, 2, 3) against control (c) were analyzed as follows: A fresh tester strain (2 µL of 1/100 dilution) was added to 100 µL of the above samples and incubated for 2, 4, and 6 hours. Bacteria were counted on nutrient agar (NA) plates and the yeast were detected on YEPD plates. After incubation, the survival percentages were determined according to the colonies-forming units (CFU).

2.9. Statistical analysis

The data were expressed as the mean and standard deviation (SD) of three replicates. The data were statistically assessed using the SPSS 23.0 program using one-way analysis of variance (ANOVA), followed by the student's t test wherein the differences were considered to be significant at $p<0.05$.

3. Results and Discussion

3.1. Chemical analysis of the oil cakes

Oil cakes usually are used either for animal feeding or discarded in the environment causing health hazards and therefore they are considered as agro-industrial wastes. Castor and wheat germ oil cakes used in this investigation were chemically analyzed for its protein, carbohydrate, fat, moisture and ash contents to estimate their nutritional values as well as their suitability for microbial production (Table 1). Both castor oil and castor oil cake are actually contain antinutritional factors, ricin and ricinine, and for this reason, castor oil cake utilized directly to obtain yeast isolate that has the capability to produce PUFAs by growing on wheat germ oil cake (Akande et al., 2011).

Table 1

Chemical analysis of the oil cakes

Oil cake	Protein %	Fat%	Ash %	Moisture%	Carbohydrate%
Castor	30.95	12.48	3.81	6.47	46.28
Wheat germ	38.58	8.630	7.053	6.5633	39.17421

The chemical analyses of the castor oil cake indicated a high content of oil (12.5%) wasted in the cake, beside its high protein (30.9%), carbohydrate (46.3%) and ash (3.8%) contents. This endows the castor oil cake its suitability for the microbial isolation. Castor seeds usually contains about 30 to 50 present oil, it can be extracted by pressing or solvent, or both (Patel et al., 2016). In fact, the first method represents a poor oil extraction level (Akande et al., 2011) and therefore, that high oil content was expected due the used pressing extraction method. In a related context, wheat germ oil cake has lower oil and carbohydrate contents (8.6 & 39.1 %, respectively) but a higher protein and ash percentage (38.6 & 7.05 %, respectively).

3.2. Isolation and evaluation of the selected yeast

The naturally grown microbes on castor oil cake under CTAB stress actually, produced colonies like yeast which were evaluated to select the best one. The isolated yeast was then molecularly identified by isolating its genetic material (DNA). Following DNA isolation and concentration assessment, primers were employed to amplify the region of the rDNA repeat unit that contains the ITS from the yeast strain's genomic DNA. Amplification however, was about 500 bp (Figure 1).

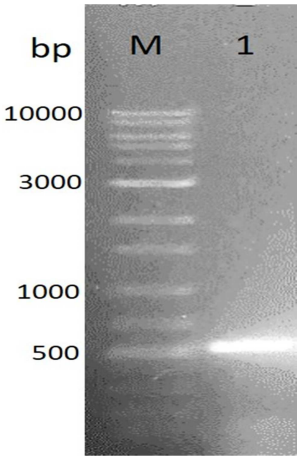

Fig. 1: Photograph of ITS-DNA amplified band for yeast strain (W5-2) isolated from castor cake (lane 1) using ITS1 and ITS4 primers against 100 bp ladder DNA marker (lane M)

Utilizing forward primer, the DNA sequencing of the purified PCR products was achieved and by applying and archive the obtained DNA sequence with the identified yeast strains in the GenBank, the accession number was as following: Seq. [organism = Saccharomyces cerevisiae] W5-2, ITS1, partial sequence; 5.8S ribosomal RNA gene, and ITS2, complete sequence; and 28S ribosomal RNA gene, partial sequence (GenBank accession number ON644539.1) as shown in Figure 2.

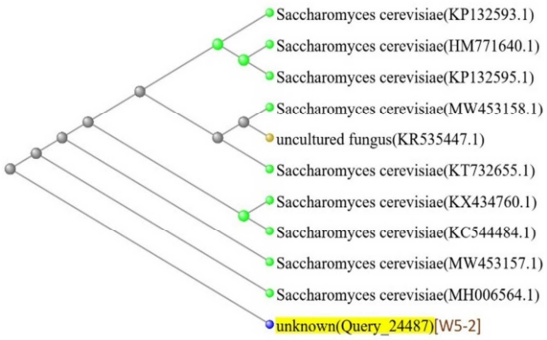

Fig. 2: A phylogenetic tree of the taxonomic position of the isolated yeast strain (W5-2) isolated from castor cake, created using the Neighbor-Joining method, based on the 16S-DNA sequences and other closely related species in database

3.3. Production of WGO and PUFAs

Cheap substrate reduces the end product price, not to mention the production process cost itself. However, the extracted WGO was as much as 14.6% from the wheat germ by-product. While, the PUFAs were produced (10.13 g/100 g substrate) from the

remained wheat germ oil cake and soybean crude oil. Actually, soybean crude oil with the remaining WGO in addition to carbohydrate contents, supply the yeast strain with carbon source, while, nitrogen source is complemented by protein from the wheat germ oil cake.

Ferreira et al., (2021) studied the production of PUFAs from Mortierella alpina utilizing SSF on polyurethane and they revealed that, linseed oil supplementation for the medium (10% w/v) increased the PUFAs production around 10-fold, compared to SSF without oil supplementation. In fact, they examined deferent vegetable oils (linseed, olive, sunflower, and castor oil) as inducers for PUFAs production whereas, the highest yield was related to linseed oil (19.3 g/100g substrate) while castor oil supplementation gave the lowest yield (0.42 g/100g substrate).

3.4. Fatty acid composition of the produced PUFAs compared to WGO

The fatty acid composition of the produced PUFAs and WGO derived from the wheat germ has been determined using GC/MS and the obtained results are listed in Table 2. It could be observed that the most represented fatty acids of the produced PUFAs and WGO extracted by cold pressing were, palmitic (9.33% & 16.28%), oleic (23.79% &17.07%), linoleic acid ω-6 (54.62% & 55.90%) and linolenic acid ω-3 (6.81% & 8.17%) respectively. The percentage of total saturated fatty acids of WGO was a relatively higher that estimated at 18.86% compared to the produced PUFAs which estimated at 14.78%. Palmitic acid (C16:0) however, was found to be the dominant fatty acid existing at concentrations slightly lower than 10% followed by stearic acid (C18:0) of total saturated fatty acids. On the other side, oleic acid (C18:1) had a higher percentage (23.79%) in the produced PUFAs compared to WGO (17.07%) of total fatty acids.

Furthermore, it should be notified that the percentage of PUFAs in WGO and produced PUFAs were almost the same and contribute 64.07% and 61.43% of total fatty acids, respectively. Linoleic acid (C18:2) presents the major fatty acid in WGO and produced PUFAs (55.90 & 54.62 %, respectively) while, α-linolenic acid (C18:3) appeared in lower ratio. Generally, linoleic (omega-6) and α-linolenic (omega-3) fatty acids are considered essential, as they cannot be synthesized in body (Moreira and Mancini-Filho, 2004). The data in Table 2 also, illustrated that the ratio of n6/n3 was slightly better in WGO than produced PUFAs (6.84/1, 8.02/1), respectively. In fact, both ratios are acceptable as the ideal n6/n3 fatty acid ratio is 4:1 according to British Nutrition Foundation, 2021. The difference in chemical composition between the produced PUFAs and WGO may be due to ability of the yeast strain Saccharomyces cerevisiae to change the medium and long chain fatty acids length in order to improved yeast growth (Duan et al., 2015). Omega-6 however, can modulate cell proliferation and migration, phagocytic capacity, and involved in the production of inflammatory mediators. They also can improve cardiovascular health when supplemented by food such as nuts and seeds (Harris et al., 2009 & Silva et al., 2018). From other side of view, omega-3 fatty acids are actually affecting the expression of pro-inflammatory cytokines gene by changing the fluidity cell membrane, cell signaling, function of membrane and cells mobility (McDaniel et al., 2008). Therefore, it is clear from the collected results, that the extracted PUFAs produced from the biotransformation of wheat germ oil cake by Saccharomyces cerevisiae were rich in healthy essential fatty acids.

Table (2)
Fatty acid composition of the produced PUFAs compared to WGO extracted by cold pressing

Fatty acids	Formula	Area Sum %	
		PUFAs	WGO
Hexadecanoic acid, methyl ester (palmitic acid)	$C_{17}H_{34}O_2$	9.33	16.28
9-Hexadecenoic acid, methyl ester (palmitoleic acid)	$C_{17}H_{32}O_2$	---	---
Methyl stearate (stearic acid)	$C_{19}H_{38}O_2$	5.45	1.03
9-Octadecenoic acid, methyl ester (oleic acid)	$C_{19}H_{36}O_2$	23.79	17.07
9,12-Octadecadienoic acid, methyl ester (linoleic acid)	$C_{19}H_{34}O_2$	54.62	55.90
9,12,15-Octadecatrienoic acid, methyl ester (linolenic acid)	$C_{19}H_{32}O_2$	6.81	8.17
Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	----	1.55
Methyl 9-eicosenoate	$C_{21}H_{40}O_2$	----	----
TSFAs		14.78	18.86
TUSFAs		85.22	81.14
PUFAs		61.43	64.07
n6/n3		8.02	6.84
S/U		0.17	0.23

3.5. The effect of the produced PUFAs and WGO on wound healing

Cytotoxicity investigation of the produced PUFAs

Cytotoxicity test is in fact a biological evaluation and screening tests intended to monitor cells of tissues used in vitro for cell growth, proliferation, and

morphological changes that are affected by exposure to a particular compound (Li et al., 2015). In order to study the effect of the produced PUFAs on wound healing compared to WGO in-vitro via HSF cells, it is necessary to investigate the cytotoxic effect of the PUFAs on the target cells. Therefore, HSF cells treated with different concentrations (0.05 -500 µg/ml) of the produced PUFAs for their viability. At the end of the incubation period the results indicated that the investigated doses were safe for HSF cells administration up to 500 µg/ml (Figure 3).

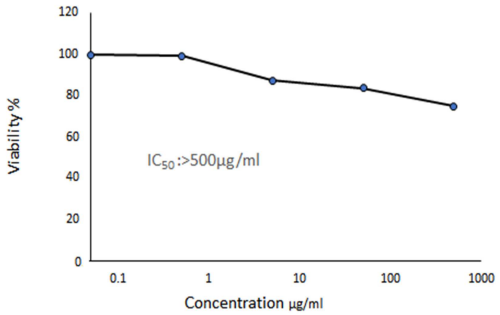

Figure (3): Dose-response curves for produced PUFAs compound, analyzed with SRB assay. Results are the means of 3 independent experiments

3.6. PUFAs and WGO effect on wound healing

Many natural products have been studied for wound healing process such as essential fatty acids (Cardoso et al., 2015; Komprda, 2018; Silva et al., 2018; Kotronoulas et al., 2020) and antioxidant compounds (Fitzmaurice et al., 2011; Agustina et al., 2021; Comino-Sanz et al., 2021) which contribute in one or more of specific function related to healing stages (Ibrahim et al., 2018). Since omega-6 fatty acids can improve cell proliferation and migration while, omega-3 can affect the pro-inflammatory cytokines gene (McDaniel et al., 2008 & Silva et al., 2018). Thus, the integration between omega 6 and 3 may affect the wound healing stages, allowing the healing process to occur in a smooth and complete manner. Accordingly, the produced PUFAs and WGO were utilized as wound healing inducer agents against the control wound healing of the HSF cells (Figure 4 & 6). Actually, the results indicated the efficiency of the produced PUFAs at the first 24hrs. However, the values were inverted as control cells healing at 48hrs was more efficient than PUFAs, but at the end of the experiment PUFAs extract as well as control cells healing were similar at 72hrs. On the other side, the WGO treatment decreased the process time of HSF cell migration comparing to control but, eventually HSF cell migration in both treated and control were completely recovered at 72hrs (Figures 5 & 7).

Kotronoulas et al., (2020) studied the effect of omega-3 PUFAs extracted from North Atlantic cod skin on wound healing of HaCat cells in vitro. They indicated that the extracted PUFAs increased the cells migration and improved the wound closure 4hrs earlier than control. Silva et al., (2018) reviewed reports concerning the roles of immune nutrients such as amino acids, minerals, and fatty acids on wound healing. They concluded that omega-6 PUFAs affected all phases of wound healing positively, they also pointed out that clinical studies are necessary for fatty acid administration whether topically or orally with optimal concentrations, and their safety.

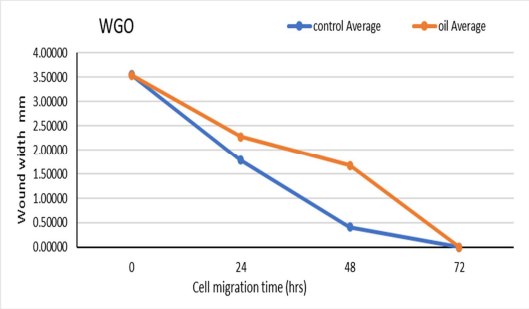

Figure (4): Wound width vs. HSF migration time during 72 hrs for PUFAs and control. The results are displayed as mean ± standard deviation.

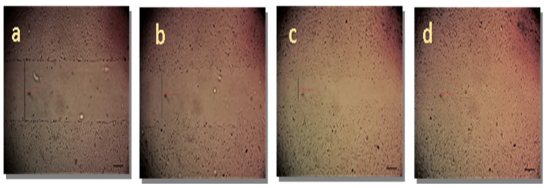

Figure (5): Microscopic images showing the effect of PUFAs on HSF migration in a wound scratch test assay, during 72 hrs where a: 0 hr; b: 24 hrs; c: 48 hrs; d: 72 hrs. The results are displayed as mean ± standard deviation

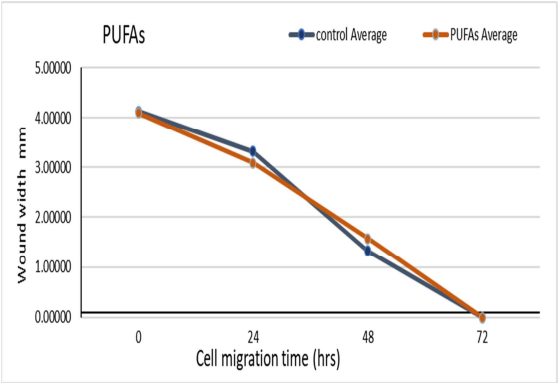

Figure (6): Wound width vs. HSF migration time during 72 hrs for WGO and control. The results are displayed as mean ± standard deviation

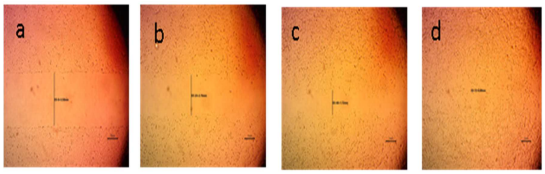

Figure (7): Microscopic images showing the effect of WGO on HSF migration in a wound scratch test assay, during 72 hrs. a: 0 hr; b: 24 hrs; c: 48 hrs; d: 72 hrs. The results are displayed as mean ± standard deviation

3.7. Antibacterial effect of extracted WGO and produced PUFAs against wound healing pathogens

Chronic wounds are usually accompanied with diverse of pathogenic microbial flora. Aerobic Gram-positive and negative pathogenic bacteria such as, *Staphylococcusspp.* and *Pseudomonas spp*. represent more than 70% of wounds pathogens (Davies et al., 2001; Hill et al., 2003; Stephens et al., 2003; Zhao et al., 2013). They are generally generating colonies before the accumulation of facultative anaerobic Gram-negative *bacilli,* such as *Escherichia coli*, *Enterobacter cloacae, Klebsiella spp,* and *Proteus spp*. which, invade the wounds and secrete cytotoxins along with enzymes, causing critical wound deterioration (Martin et al., 2010; Bessa et al., 2015; Das et al., 2016). Therefore, the effect of WGO and produced PUFAs against *Staphylococcus aureus NRRL B-767* and *Pseudomonas aeruginosa ATCC 9027* was investigated. The results in figures 8 & 9 revealed that the PUFAs were superior to WGO in growth inhibition of both strains. It was also found that, PUFAs were more inhibitive for the growth of *S. aureus* than *P. aeruginosa*. PUFAs were able to kill about 25% of the *S. aureus* population compared to WGO which inhibits about 15 % only of the same strain after 6 hrs. While, *P. aeruginosa* was inhibited by 14.6 and 12.2% after 6hrs, utilizing PUFAs and WGO, respectively. The seriousness of microbial infection lies in the bacteria and its cytotoxins secretions upon wounds which prolong the elevation of pro-inflammatory cytokines causing elongation of the inflammatory phase. In fact, wound as a response to such action, may fail to heal and enter to the chronic state (Edwards & Harding, 2004; Menke et al., 2007). Accordingly, there is a great necessity to obtain antimicrobial agents to deal with wound infections (Eshlak, 2019). The collected results, indicated that not only produced PUFAs manage to control wound healing efficiently specially at the first 24hrs but also, indicated their ability to inhibit 15 and 25% of the most common wound infection bacteria *P. aeruginosa* and *S. aureus*, respectively.

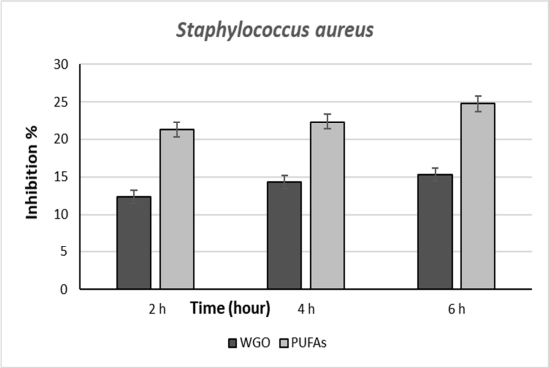

Figure (8): Antimicrobial activity of WGO and produced PUFAs against *Staphylococcus aureus.* Error bars indicate standard deviations from triplicate measurements

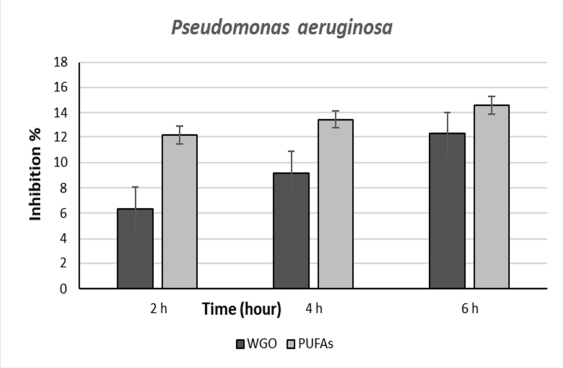

Figure (9): Antimicrobial activity of WGO and produced PUFAs against *Pseudomonasaeruginosa.* Error bars indicate standard deviations from triplicate measurements

4. Conclusions

In this study, at the environmental level, wheat germ, which is one of the agro-industrial wastes that causes environmental damage, has been utilized to obtain wheat germ oil, and the remaining oil cake was used to obtain another valuable product, namely PUFAs, that obtained by microbial conversion using *Saccharomyces cerevisiae.* The yeast strain was locally isolated from another waste (castor oil cake). Accordingly, the study achieved a dual economic advantage, as both wheat germ oil and PUFAs were obtained from the same waste. The efficiency of the produced PUFAs have been proven to act as wounds healing agent in the first 24hrs. Also, the produced PUFAs were able to inhibit both most common wound infectious bacteria *P.aeruginosa* and *S. aureus* by 15 and 25%, respectively.

5. Acknowledgments

The authors express their thanks to the National Research Centre, Egypt for itssupport and funding during this work under the Project no. 12020116.

6. Conflict of interest:

The author declares no conflict of interest

7. References

A.O.A.C. (1980). Analysis of the Association of Official Agricultural Chemists, 13th ed., Washington, D.C., USA.

Agustina, S., Aidha, N. N., Oktarina, E., & Kurniati, N. F. (2021). Evaluation of Antioxidant and Wound Healing Activities of Spirulina sp. Extract. *Egyptian Journal of Chemistry*, *64*(8), 1-2.

Aita, B. C., Spannemberg, S. S., Kuhn, R. C., & Mazutti, M. A. (2018). Production of Polyunsaturated Fatty Acids by Solid State Fermentation. *Principles and Applications of Fermentation Technology*, 217.

Akande, T. O., Odunsi, A. A., & Adedeji, O. S. (2011). Toxicity and nutritive assessment of castor (Ricinus cummunis) oil and processed cake in rat diet. *Asian Journal of Animal Sciences*, *5*(5), 330-339.

Al-Kashef, A., Shaban, S., Nooman, M., & Rashad, M. (2018). Effect of fungal glycolipids produced by a mixture of sunflower oil cake and pineapple waste as green corrosion inhibitors. *Journal of Environmental Science and Technology*, *11*(3), 119-131.

Ancuța, P., & Sonia, A. (2020). Oil press-cakes and meals valorization through circular economy approaches: A review. *Applied Sciences*, *10*(21), 7432.

Arslan, D., Demir, M. K., Acar, A., & Arslan, F. N. (2020). Investigation of wheat germ and oil characteristics with regard to different stabilization techniques. *Food Technology and Biotechnology*, *58*(3), 348.

Bessa, L. J., Fazii, P., Di Giulio, M., & Cellini, L. (2015). Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. *International wound journal*, *12*(1), 47-52.

Boekhout T, Kurtzman CP, apos, donnell K, & Smith MYT (1994). Phylogeny of the Yeast Genera Hanseniaspora (Anamorph Kloeckera), Dekkera (Anamorph Brettanomyces), and Eeniella as Inferred from Partial 26S Ribosomal DNA Nucleotide Sequences. 44: 781-786.

Brandolini A, Hidalgo A. Wheatgerm: Not only a by-product. Int J Food Sci Nutr. 2012;63(Suppl. 1):71-4. https://doi.org/10.3109/09637486.2011.633898

British Nutrition Foundation, 2021 https://www.nutrition.org.uk/healthy-sustainable diets/fat/?level=Health%20professional

Çalışkan Koç, G., & Özçıra, N. (2019). Chemical composition, functional, powder, and sensory properties of tarhana enriched with wheat germ. *Journal of Food Science and Technology*, *56*(12), 5204-5213.

Cardoso, M. D. P. C., Albuquerque, A. D. O., Girão, V. C. C., Pompeu, M. M. D. L., Silva, C. I. S. M., Azevedo, O. G. R. D., ... & Vasconcelos, P. R. L. D. (2015). Preconditioning with mono and polyunsaturated fatty acids and low-intensity electrical stimulation. Effects on skin repair in rats. *Acta Cirúrgica Brasileira*, *30*, 107-114.

Comino-Sanz, I. M., López-Franco, M. D., Castro, B., & Pancorbo-Hidalgo, P. L. (2021). The role of antioxidants on wound healing: A review of the current evidence. *Journal of Clinical Medicine*, *10*(16), 3558.

Das, S., Lindemann, C., Young, B. C., Muller, J., Österreich, B (2016). Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. *Proceedings of the National Academy of Sciences of the United States ofAmerica*, *113*(22), E3101-10.

Davies, C. E., Wilson, M. J., Hill, K. E., Stephens, P., Hill, C. M., Harding, K. G., & Thomas, D. W. (2001). Use of molecular techniques to study microbial diversity in the skin: chronic wounds reevaluated. Wound repair and regeneration, 9(5), 332-340.

Duan, L. L., Shi, Y., Jiang, R., Yang, Q., Wang, Y. Q., Liu, P. T., ... & Yan, G. L. (2015). Effects of adding unsaturated fatty acids on fatty acid composition of Saccharomyces cerevisiae and major volatile compounds in wine. *South African Journal of Enology and Viticulture*, *36*(2), 285-295.

Edwards R, Harding KG (2004). Bacteria and wound healing. *Curr OpinInfect Dis* 17:91-96.

El-Hamidi, M., & Zaher, F. A. (2018). Production of vegetable oils in the world and in Egypt: an overview. *Bulletin of the National Research Centre*, *42*(1), 1-9.

Eshlak, M. (2019). *Antibacterial activity of polyunsaturated fatty acids related to chronic wound infections*. University of Salford (United Kingdom).

Ferreira, M., Fernandes, H., Peres, H., Oliva-Teles, A., Belo, I., & Salgado, J. M. (2021). Polyunsaturated fatty acids production by solid-state fermentation on polyurethane foam by Mortierella alpina. *Biotechnology Progress*, *37*(3), e3113.

Fitzmaurice, S. D., Sivamani, R. K., & Isseroff, R. R. (2011). Antioxidant therapies for wound healing: a clinical guide to currently commercially

available products. *Skin pharmacology and physiology*, *24*(3), 113-126.

Gili, R. D., Irigoyen, R. M. T., Penci, M. C., Giner, S. A., & Ribotta, P. D. (2018). Wheat germ thermal treatment in fluidised bed. Experimental study and mathematical modelling of the heat and mass transfer. *Journal of Food Engineering*, *221*, 11-19.

Harris, W. S., Mozaffarian, D., Rimm, E., Kris-Etherton, P., Rudel, L. L., Appel, L. J., ... & Sacks, F. (2009). Omega-6 fatty acids and risk for cardiovascular disease: a science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. *Circulation*, *119*(6), 902-907.

Hill, K. E., Davies, C. E., Wilson, M. J., Stephens, P., Harding, K. G., & Thomas, D. W. (2003). Molecular analysis of the microflora in chronic venous leg ulceration. *Journal of medical microbiology*, *52*(4), 365-369.

Ibrahim, N. I., Wong, S. K., Mohamed, I. N., Mohamed, N., Chin, K. Y., Ima-Nirwana, S., & Shuid, A. N. (2018). Wound healing properties of selected natural products. *International journal of environmental research and public health*, *15*(11), 2360.

Jonkman, J. E., Cathcart, J. A., Xu, F., Bartolini, M. E., Amon, J. E., Stevens, K. M., & Colarusso, P. (2014). An introduction to the wound healing assay using live-cell microscopy. *Cell adhesion & migration*, *8*(5), 440-451.

Jukes TH, & Cantor CRJMpm (1969). Evolution of protein molecules. 3: 21-132.

Komprda, T. (2018). Effect of n-3 long-chain polyunsaturated fatty acids on wound healing using animal models-a review. *Acta Veterinaria Brno*.

Kotronoulas, A., Jónasdóttir, H. S., Sigurðardóttir, R. S., Halldórsson, S., Haraldsson, G. G., & Rolfsson, Ó. (2020). Wound healing grafts: Omega-3 fatty acid lipid content differentiates the lipid profiles of acellular Atlantic cod skin from traditional dermal substitutes. *Journal of Tissue Engineering and Regenerative Medicine*, *14*(3), 441-451.

Li B, Zhao L, Chen H, Sun D, Deng B, Li J, *et al.* Inactivation of lipase and lipoxygenase of wheat germ with temperature-controlled short wave infrared radiation and its effect on storage stability and quality of wheat germ oil. PLoS ONE. 2016;11(12):e0167330.

Li, W., Zhou, J., & Xu, Y. (2015). Study of the in vitro cytotoxicity testing of medical devices. *Biomedical reports*, *3*(5), 617-620.

Main K.A., Mikelis C.M., Dog C.L. (2019) In Vitro Wound Healing Assays to Investigate Epiderma Migration. In: Turksen K. (eds) Epidermal Cells. Methods in Molecular Biology, vol 2109. Humana, New York, NY

Marti A, Torri L, Casiraghi MC, Franzetti L, Limbo S, Morandin F, *et al.* Wheat germ stabilization by heat-treatment or sourdough fermentation: Effects on dough rheology and bread properties. LWT - Food Sci Technol. 2014;59(2):1100 6. https://doi.org/10.1016/j.lwt.2014.06.039

Martin, J. M., Zenilman, J. M., & Lazarus, G. S. (2010). Molecular Microbiology: New Dimensions for Cutaneous Biology and Wound Healing. *Journal of Investigative Dermatology*, *130*(1), 38– 48.

Martinotti S., Ranzato E. (2019) Scratch Wound Healing Assay. In: Turksen K. (eds) Epidermal Cell Methods in Molecular Biology, vol 2109. Humana, New York, NY

McDaniel, J. C., Belury, M., Ahijevych, K., & Blakely, W. (2008). Omega-3 fatty acids effect on wound healing. *Wound Repair and Regeneration*, *16*(3), 337-345.

Menke, N. B., Ward, K. R., Witten, T. M., Bonchev, D. G., & Diegelmann, R. F. (2007). Impaired wound healing. *Clinics in dermatology*, *25*(1), 19-25.

Moreira,A.V.B.and Mancini-Filho,J.(2004).Influence of spices phenolic compounds on lipoperoxidation and lipid profile of rats tissues.Rev.Nutr.,17(4):411-424.

Nooman, M. U., Mahmoud, M. H., Al-Kashef, A. S., & Rashad, M. M. (2017). Hypocholesterolemic impact of newly isolated sophorolipids produced by microbial conversion of safflower oil cake in rats fed high-fat and cholesterol diet. *Grasas y Aceites*, *68*(3), e212-e212.

Patel, V. R., Dumancas, G. G., Viswanath, L. C. K., Maples, R., & Subong, B. J. J. (2016). Castor oil: properties, uses, and optimization of processing parameters in commercial production. *Lipid insights*, *9*, LPI-S40233.

Rashad MM, Al-Kashef A, Nooman M, El-din-Mahmoud AJRJoP, Biological, & Sciences C (2014b). Co-utilization of motor oil waste and sunflower oil cake on the production of new sophorolipids by Candida bombicola NRRL Y-17069. 5: 1515-1528.

Rashad MM, Nooman M, Ali M, Al-Kashef A, & Mahmoud AJGyA (2014a). Production, characterization and anticancer activity of Candida bombicola sophorolipids by means of solid state fermentation of sunflower oil cake and soybean oil. 65: e017-e017.

Rodriguez L.G., Wu X., Guan JL. (2005) Wound-Healing Assay. In: Guan JL. (eds) Cell Migration. Methoc in Molecular Biology™, vol 294. Humana Press

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017 ImageJ2: ImageJ for the next generation of scientific image data. BMC bioinformatics, 18(1), 529.

Saitou N, & Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution 4: 406-425.

Sarker, A. K., Saha, D., Begum, H., Zaman, A., & Rahman, M. M. (2015). Comparison of cake compositions, pepsin digestibility and amino acids concentration of proteins isolated from black mustard and yellow mustard cakes. *AMB Express*, *5*(1), 1-6.

Sharma, S., Verma, M., & Sharma, A. (2013). Utilization of non edible oil seed cakes as substrate for growth of Paecilomyces lilacinus and as biopesticide against termites. *Waste and Biomass Valorization*, *4*(2), 325-330.

Silva, J. R., Burger, B., Kühl, C., Candreva, T., Dos Anjos, M. B., & Rodrigues, H. G. (2018). Wound healing and omega-6 fatty acids: From inflammation to repair. *Mediators of inflammation*, *2018*.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, et al. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82(13): 1107-1112

Starzyńska-Janiszewska, A., Stodolak, B., Mickowska, B., & Socha, R. (2021). Fermentation with edible Rhizopus strains as a beneficial alternative method in wheat germ cake processing. *Journal of Cereal Science*, *102*, 103309.

Stephens, P., Wall, I. B., Wilson, M. J., Hill, K. E., Davies, C. E., Hill, C. M., ... & Thomas, D. W. (2003). Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro. *British Journal of Dermatology*, *148*(3), 456-466.

Ummalyma, S. B., Sindhu, R., Binod, P., Pandey, A., & Gnansounou, E. (2019). Production and Applications of Polyunsaturated Fatty Acids. *High Value Fermentation Products Volume 2*, 2109.

Üstun G, Kent L, Cekin N, Civelekoglu H. 1990. Investigation of the technological properties of *Nigella sativa* (black cumin) seed oil. *J. Am. Oil. Chem. Soc*. 67, 958–960. https://doi.org/10.1007/BF02541857

Wickerham LJ (1951) *Taxonomy of yeasts*. US department of agriculture.

Zhao, G., Usui, M. L., Lippman, S. I., James, G. A. & Olerud, J. E. (2013). Biofilms and Inflammation in Chronic Wounds. *Advances in Wound Care*, *2*(7), 389–399.