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Antibacterial, antioxidant and anticancer of fermentation by Bacillus subtilis on bagasse and wheat bran

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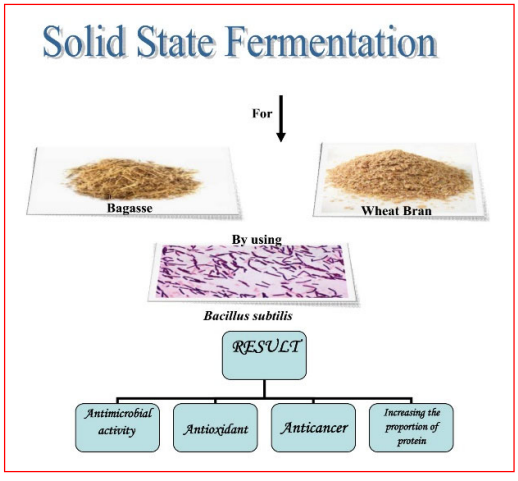
A B S T R A C T

Bagasse and wheat bran are agrowaste and cause different environmental problems. These compounds contain highly valuable compounds that can be recycled by microorganisms. This work was carried out to evaluate the effect of fermentation by *Bacillus subtilis* on biological activity and chemical components of bagasse and wheat bran. The results demonstrated the antimicrobial activity of fermented wheat bran and bagasse against eight selected microbial pathogens exhibited high activity of fermented wheat bran and mix, fermented bagasse showed less antimicrobial activity. Fermented wheat bran and bagasse samples had the highest antioxidant activity values (16.45 and 14.94 %) in comparison with the unfermented samples (6.20 and 3.97 %) respectively. Concentration of protein, ash, oil, and carbohydrates in fermented wheat bran were 5.34 %, 7.30%, 2.36% and 10.6%, whereas in bagasse they were 2.45%, 1.68%, 1.51% and 3.25 respectively. The moisture contents in bagasse 73.1% were more than in wheat bran 63.88%.



Graphical Abstract

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1. Introduction

Wheat, a type of grass plant, is second only to rice as the main human food crop. Commercially, *Triticum sativum* and *Triticum turgidum* durum (hard wheat, mainly used in pasta products) are with high importance.[1] The wheat grain or 'caryopsis', which is harvested for human nutrition, is composed of a number of different tissues: the germ (or embryo); the endosperm, which is packed with starch grains to provide energy for germination; the thick cell-walled aleurone layer, encasing the endosperm; and the pericarp. The bran fractions consist of the pericarp, testa, and hyaline and aleurone layers. By weight, the wheat caryopsis is composed of an outer branny husk (14–16 % of the grain), the germ or embryo (2–3%), and the central endosperm (mainly starch: 81–84 %).[2]

In recent years, there has been an increasing trend toward the more efficient use of agro-industrial waste, including sugarcane bagasse. Various processes and products that use sugarcane bagasse as support have been reported. These include power generation, the production of paper, and the manufacture of products based on fermentation.[3] Different techniques were used in many manufacturing areas, one of them is silage production. Solid substrates include rice straw, corn stalks, bagasse, and wheat bran. Solid state fermentation achieved by many microorganisms' molds and bacteria.[4] *Bacillus subtilis*, known also as the hay Bacillus or grass Bacillus, is a Gram-positive, catalase-positive bacteria, found in soil and the gastrointestinal tract of ruminants, humans and marine sponges.[5]

As a member of the genus Bacillus, *B. subtilis* is rod-shaped, and can form a tough, protective endospore, allowing it to tolerate extreme environmental conditions. *B. subtilis* has historically been classified as an obligate aerobe, though evidence exists that it is a facultative anaerobe. *B. subtilis* is considered the best studied Gram-positive bacterium and a model organism to study bacterial chromosome replication and cell differentiation. It is one of the bacterial champions in secreted enzyme production and used on an industrial scale by biotechnology companies.[5]

Bacillus subtilis is one of the most important microorganisms beneficial in improving the meat quality and antioxidant capacity of animals. Antioxidant is a molecule that can slow or stop the oxidation, or electron transfer, of other molecules. Antioxidants can be found in food. Antioxidants are molecules that relieve oxidative stress by preventing the formation and oxidation of free radicals.[6] Antioxidants donate one of their electrons or hydrogen to free radicals, stopping their chain reaction.[7] *Bacillus subtilis* fermentation is an alternative for the production of phenolic and peptide compounds and enhancing antioxidant activity of the defatted wheat germ. In the past, there have been major concerns about the anti-nutritional effects of some phenolic family members. An example is the case of high concentration of tannins in animal feeds when certain sorghum species are used. However, contemporary thinking focuses more on the beneficial effects of phenol, and this has shed new light in the use of these compounds as alternative additives in animal feed. Phenolic compounds including simple phenols and phenolic acids are an enormous group comprising more than 8,000 diverse compounds of plant secondary metabolic products. A key feature of phenol is its antioxidant activity. However, some phenols may have additional beneficial properties applicable to animal intestinal health, such as antimicrobial activity, antioxidants or anticancer. All these diverse properties are the driving force for the presence of these bioactive compounds in plants, where they act as pigments, structural components, defense compounds, antioxidants.[8]

2. Results and Discussion

2.1.1 Chemical composition of fermented and non-fermented wheat bran and bagasse by Bacillus subtilis fermented

Table 1 shows that the chemical composition of raw materials before and after fermentation. The results clear that the chemical contents of wheat bran, bagasse and mixture after and before fermented by *B. subtilis.*

Table 1. Chemical composition of fermented and non-fermented Wheat bran and Bagasse by *Bacillus subtilis* Fermented.

	Unfermented		Fermented		
Items	Wheat bran	Bagasse	Wheat bran	Bagasse	Wheat bran and Bagasse mix
Moisture	9.40+0.25	10.63+0.04	73.28+1.35	83.73+0.97	87.30+0.68
Ash	4.95+0.04	3.54+0.38	7.30+0.45	1.68+0.01	2.70+0.20
Oil	5.47+0.48	3.10+0.23	7.83+0.38	4.61+0.04	6.10+0.70
Protein	9.54+0.16	2.45+0.00	14.88+1.23	6.06+1.24	14.29+0.19
Carbohydrate	80.04+0.26	90.90+0.15	69.98+1.16	87.65+1.30	76.90+1.11

Protein content in Wheat bran was (14.88, and 9.54%), bagasse (2.45 and 6.06) before and after fermentation respectively, meanwhile the protein content in mixture was 14.29%. The best percentage of carbohydrate contents was obtained with bagasse (90.90%) before fermentation. Meanwhile, the best oil contents was (7.83%) with fermented wheat bran. Other result showed that the main limitations of plant proteins are the deficiencies in certain essential amino acids and minerals, and the presence of anti-nutritional factors and complex carbohydrates.[9] Microorganisms can be cultivated on agro-industrial products with production of large amounts of cells rich in proteins that commonly contain all the essential amino acids, in addition to favorably high vitamin and mineral levels.[10] For experiment I, after every 12 h and for experiments II and III,

after every 24 h, the fermented products were dried to a constant weight, ground to <1mm size and proximate composition analyses were carried out. Prior to the start of the three experiments, initial proximate analyses were done for all the substrates. Chemical analysis of unfermented and fermented samples included moisture content, crude protein, crude ash, crude fat and nitrogen free extract (NFE).[11] Amino acids profile was determined using HPLC (Waters India Ltd.) after acid hydrolysis. Tryptophan was determined spectro-photometrically after alkali hydrolysis.[11] The results were analyzed by two-way ANOVA.

2.1.2 Phenolic compounds and antioxidant activity of fermented and unfermented samples

Table 2 shows the activity of phenolic compounds and antioxidants of wheat bran and bagasse fermentation by *B.subtilis*. According to the obtained results Phenolic compounds of fermented wheat bran and bagasse(mixture) exhibited highest concentration 0.7350 mg/g, where's fermented of wheat bran was 0.5149 mg/gm, and fermentation of bagasse was 0.47.29 mg/g, the results showed that the fermentation of mixture better than of fermentation alone compared with unfermented samples 0.3503 and 0.2743 mg/g, respectively.

On the other hand, the antioxidant activity of fermented wheat bran with bagasse (mixture) exhibited highest activities percentage 16.89, fermentation of wheat bran was 16.45%, while fermentation of bagasse was 14.94%, the results showed that there are no significant difference in results each fermentation than fermentation with mixture compared with unfermented samples 6.20 and 3.97%, respectively.

Martins,[12] showed that the agro-industrial residues of vegetables and cereals such as wheat bran, bagasse, straw, corn cob, among others are lignocellulosic materials mainly composed of cellulose, hemicellulose and lignin. The lignin fraction of these materials contains numerous phenolic compounds, mainly acids such as ferulic, coumaric, syringic and hydroxybenzoic, which can also be recovered by SSF. Since fungi grow on these residues, use the polysaccharides after lignin degradation in order to grow and reproduce. The release of ferulic acid from agricultural byproducts by enzymatic methods has been increasingly researched, with most studies using yeast as an enzyme source. [12] Ferulic acid has commercial potential and may be applied as a natural precursor of vanillin, natural antioxidant, preservative agent in food products, anti-inflammatory agent and photo-shield.[13] The antioxidant activity of the phenolic compounds was evaluated by inhibiting free radical DPPH, expressed in terms of the ability to reduce/sequester the free radical. Compared to others, this is a widely used method to evaluate the antioxidant capacity in a short time interval.[14] Ferulic acid was the main phenolic compounds found in fermented and unfermented rice bran.[15]

Table 2. Phenolic compound and antioxidant activity of fermented substrate of Wheat bran and Bagasse.

Items	Unfermented		Fermented		
	Wheat bran	Bagasse	Wheat bran	Bagasse	Wheat bran and Bagasse mix
Phenolic compound mg/g	0.3503+1.11	0.2743+0.74	0.51.49+3.09	0.4729+0.12	0.7350+0.86
Antioxidant activity%	6.20+1.93	3.97+1.05	16.45+0.04	14.94+0.01	16.89+0.005

2.1.3 Antimicrobial activity of fermented wheat bran and bagasse

Table 3 and **Fig. 1** show that the antimicrobial activity of wheat bran, bagasse and mix fermentation by *Bacillus subtilus*. According to the obtained results antimicrobial activity showed that mixture (wheat bran and bagasse) exhibited highest activity with clear zone 20.2 mm against *Staphylococcus aureus*, 20mm against *Candida albicans*, *Bacillus anthracoid* and *Klebsiella pneumonia,* 18 mm against *Fusarium lattium,* against *Pseudomonas aeruginosa*, 16mm against *proteus vulgaris*, 12 mm against *Saccharomyces cerevisiae*, antimicrobial activity of wheat bran was 15mm against *Fusarium lattium*, 18 mm against *Candida albicans* and 12 mm against *Bacillus anthercoid*, 12 mm against *Saccharomyces cerviaceae*, 18mm *Staphylococcus aureus*, 16 mm against *Proteus vulgaris*, 15 mm against *Pseudomonas aeruginosa*, 13 mm against *Klebsiella pneumonia*, while antimicrobial activity of bagasse was14 mm against *Candida albicans*, 15.17 mm against *Staphylococcus aureus*, 15 mm against *Klebsiella pneumonia*, 20 mm against *proteus vulgaris*, 11mm against *Saccharomyces cerevisiae* and not diviation against *Bacillus anthracoid, Pseudomonas aeruginosa* and *Fusarium lattium*.

Table 3. Antimicrobial activity of fermented wheat bran and Bagasse determined by clear zone (mm).

Tested Microorganisms	Wheat bran	Bagasse	(Wheat bran and bagasse) mixer
Staphylococcus aureus	**18**	**15.17**	**20.20**
Klebsiella pneumoniae	**13**	**15**	**20**
proteus vulgaris	**16**	**20**	**16**
Bacillus anthracoid	**12**	**n.d**	**20**
Pseudomonas aeruginosa	**15**	**n.d**	**18**
Saccharomyces cerevisiae	**12**	**11**	**12**
Candida albicans	**18**	**14**	**20**
Fusarium lattium	**15**	**n.d**	**18**

*n.d: not detected

The results showed that fermentation of mixture better than of fermentation with wheat bran alone and more better than of fermentation with bagasse alone. Other results showed that Bacillus genus is successfully used in solid-state fermentation to improve antimicrobial activity of fermented food.[16] Who Rochín-Medina cleared that SSF enhanced antibacterial activity. That phenomenon could be explained by the fact that *Bacillus sp*. strains could metabolize fiber which releases phenolic compounds as a result of lignocellulolytic activity.[16]

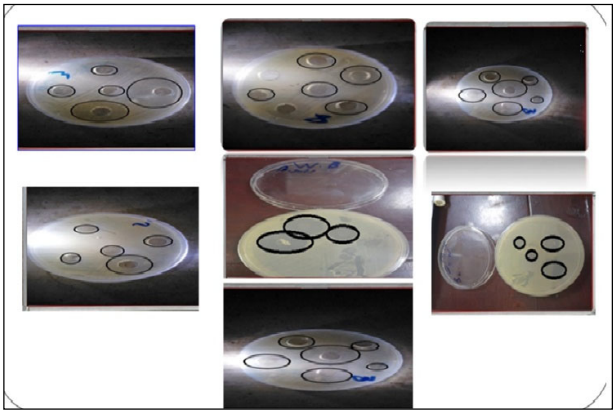

Fig 1. Antimicrobial activity and inhibition zone

2.1.4 Anticancer activities of wheat bran extract and on breast cancer cell MCF-7

The results obtained in **Table 4** and **Fig. 2** denote the effect of different concentrations of wheat bran extract on breast cancer cell MCF-7, and hence to obtain the half maximal inhibitory concentration (IC50) for wheat bran extract. Results cleared that the (IC50) was 126.5182 mg/ml. The effect of wheat bran extract against cancer cell MCF-7 was showed in **Figs (3-5)** that represent a photomicrograph showed cellular shrinkage (green arrow), irregular cell membrane (red arrows) and peripheral condensation of chromatin (Yellow arrows) of cancer cell MCF-7. **Fig. 6** represent the cancer cell MCF-7 control without any treatments, that show regular cells with hyper chromatic nuclei and nuclear pleomorphism Cells MCF-7 that treated by of wheat bran extract revealed early apoptotic features of cellular and nuclear shrinkage, irregular cellular and nuclear membranes and peripheral chromatin condensation. Late apoptotic features of nuclear fragmentation and cell membrane blebbing could also be detected. Necrotic cells and necrotic debris were also features in this group.

Table 4. Cytotoxicity effect of wheat bran extract on breast cancer cell MCF-7.

Concentration mg/ml	0.235±0.008	0.47±0.003	0.95±0.006	1.9±0.003	3.9±0.002	7.8±0.004	15.6±0.004	62.5±0.007	125±0.004	250±0.004	500±0.003	1000±0.0039
Absorbance	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.27	0.17	0.14	0.12	0.12
Viability %	99.50	99.21	99.71	100.29	99.71	99.86	99.79	76.21	49.36	38.57	35.07	33.50
IC50 mg/ml	126.5182											
Negative control	0.35	0.35	0.34	0.33	0.35	0.36	0.34	0.35	0.35	0.36	0.35	0.38
Viability of control %	100 %											

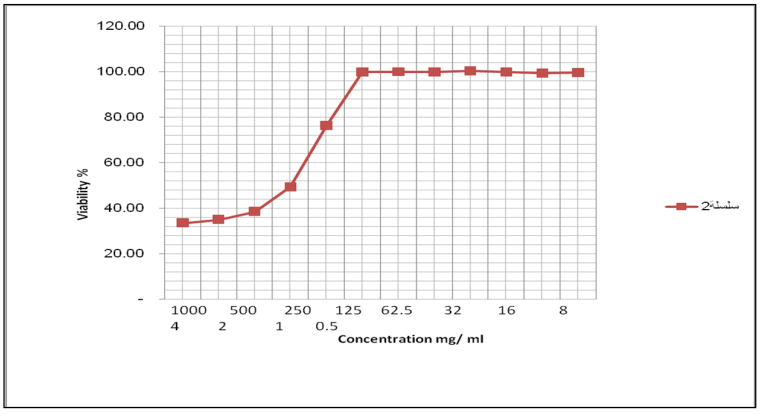

Fig. 2. The percentage of cellular viability

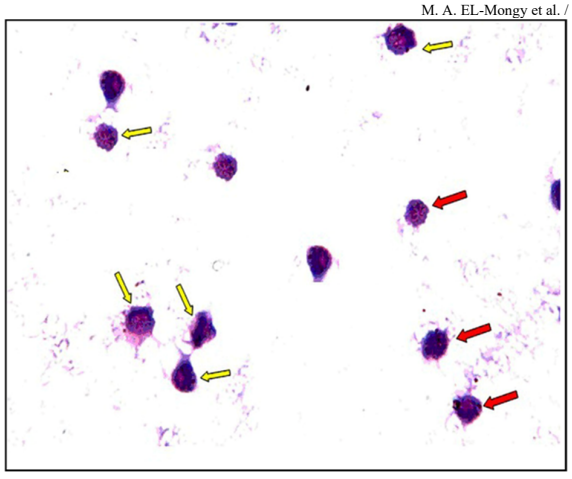

Fig. 3. A photomicrograph showing cellular shrinkage (green arrow), irregular cell membrane (red arrows) and peripheral condensation of chromatin (Yellow arrows) (H and E, original magnification 100X, Oil).

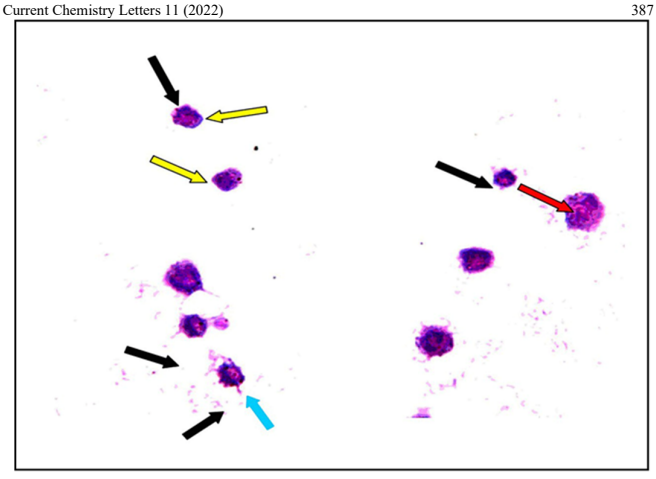

Fig. 4. A photomicrograph showing cellular shrinkage with irregular cell membrane (yellow arrows), peripheral condensation of chromatin (black arrows), membrane blebbing (blue arrow), necrotic cell with mixed euchromatin and heterochromatin (red arrow) (H and E, original magnification 100X, Oil).

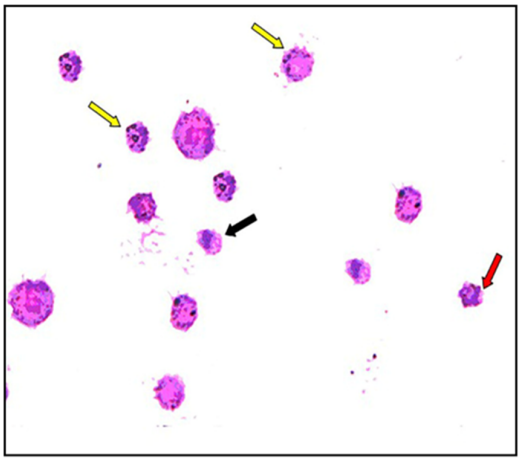

Fig. 5. A photomicrograph showing cellular (red arrow) and nuclear shrinkage (back arrow) and peripheral condensation of chromatin (Yellow arrows) (H and E, original magnification 100X, Oil).

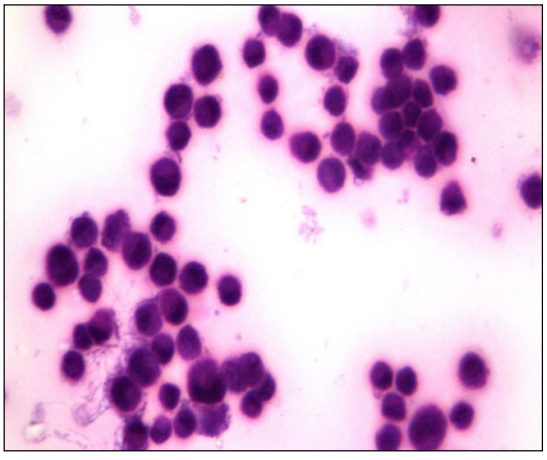

Fig. 6. MCF7 to micrograph showing regular cells with hyper chromatic nuclei and nuclear pleomorphism (H and E, Original magnification 100X, Oil) (Control)

3. Conclusion

The range of variables during solid state fermentation in antimicrobial activity, antioxidant, anticancer and chemical components changes compared with unfermented samples. Fermented wheat bran and mixture with bagasse showed antimicrobial activities against *Staphylococcus aureus*, *Klebsiella pneumonia, Proteus vulgaris, Bacillus anthracoids, Pseudomonas aurogionosa, Sacharomyces ceviceae, Candida albicans,* and *Fusarium sp*, but in case of fermented bagasse showed less antimicrobial activity in comparisons with fermented wheat bran and mixture. Wheat bran fermentation alone had high results in increasing the proportion of protein, antioxidant, antimicrobial and anticancer. While the effect of the wheat bran mixture and bagasse was similar or least to wheat bran fermentation alone were close to the results of the mixture when used as antioxidants. So this study recommends using wheat bran alone after fermentation to increase the proportion of protein and as an antioxidant, antimicrobial and anticancer.

4. Experimental

4.1 Materials and methods

Basal substrate used in research are bagasse and wheat bran were purchased from local market (Sohag, Egypt). Microbial culture of *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Proteus vulgaris, Bacillus anthracoids, Pseudomonas aurogionosa, Sacharomyces ceviceae, Candida albicans* and *Fusarium* sp microorganism strains were

obtained from Microbiology department, faculty of Science, Al Azhar University, Assiut branch . *Bacillus subtilis* strain were stored and activated on nutrient broth for 16 hr for solid state fermentation (SSF) as described in Torino data.[17]

Table 5. Characteristics of B. subtilis isolate.

Test	Result	Test	Result
Gram stain	+	Hydrogen sulfide production	_
Shape	Rod	Indole production	_
Endospore	+	Acid from manitol	+
Motility	+	Nitrate reduction	+
Catalase	+	Oxidase	+
Citrate (simmons)	+	Phenylalanine deaminase	_
Acid from sucrose	+	Acid from lactose	_
Gas from glucose	+	Urea hydrolysis	_
Gelatin hydrolysis	+	Voges-proskaeur	+
Acid from glucose	+		

4.2 Inoculums Preparation

Bacterial suspension (1ml) was prepared to inoculate a 100 ml suspension of nutrient broth (5g/100ml) after sterilization at 121° C for or 15 min. The cultures were shaken on a rotary shaker overnight at room temperature. From this 5 ml were used as starters in the following experimental work.

4.3 Preparation of solid state fermentation:

The substrates including wheat bran and bagasse were grinded well after grinding pretreatment at 0.1 NaOH for 3 hrs and then washing with sterilized distilled water until reach to (pH 6) and drying well and sterilized it in autoclave at 121°C for 30 min. Then inoculated it with 10 % (v/w) inoculums containing *Bacillus subtilis* to make plant wastes fermented by *Bacillus subtilis* mixed carefully under sterile conditions and incubated at 37°C for 48 h. All experiments were performed in triplicate.

4.3.1 Chemical composition of fermented and non-fermented samples:

Moisture, protein, fat and ash contents were determined according to official methods as described in .[18]Carbohydrates were determined by method of (9). [9]

4.3.2 Determination of total Phenolic

Samples (0.5gm) were extracted by 25 ml ethanol 50% ml shacked for 2 hours, then centrifuged at 3000 rpm for 20 min. Total phenolic compounds (TPC) were calculated in the ethanolic extracts, according to the Folin– Ciocalteu method.[19] with some modification as follow: A 100 µL aliquot of ethanolic extract was mixed with 900 µL of 10 fold Folin–Ciocalteu phenol reagent (diluted 1:10 with distilled water) and was allowed to stand for 5 min at room temperature; 0.75 µL of 7% sodium bicarbonate solution was added to the mixture and vortexed for 30 s, and allowed to stand for 90 min at room temperature. The absorbance was measured at 725 nm using a spectrophotometer (6505 UV/Vis, Jenway LTD., Felsted, Dunmow, UK). A calibration curve of gallic acid (ranging from 0 to 1.00 mg/mL) was prepared and tested under similar conditions. All values were expressed as mean (mg Gallic acid equivalents/g of dry weight for 3 replications.

4.3.3 Determination of antioxidant activity

Samples were extracted using methods described by Zielijski. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was carried out according to the method described by Lee, with some modifications. The stock reagent solution (10-3M) was prepared by dissolving 22 mg of (DPPH) in 50 ml of methanol and stored at 20°C until use. The working solution (6 x 10-5M) was prepared by mixing 6 mL of stock solution with 100 mL of methanol to obtain an absorbance value of 0.8±0.02 at 515 nm, (control) as measured using a spectrophotometer. Extract solution of tested samples (0.1 ml) were vortexes for 30 s with 3.9 ml of DPPH solution and left to react for 30 min, the absorbance was measured at 515 nm. Scavenging activity was calculated as follows: DPPH radical scavenging activity (%) = [(Ab control - Ab sample) / Ab control] X 100 Where Ab is the absorbance at 515 nm.

4.3.4 Determination of antimicrobial activity

All samples were minced and extracted with 10 times methanol (w:v) at room temperature for 5 h (repeated three times) and was then filtered through Whatman No. 4 filter paper. The methanol extracts of each sample were concentrated at 40

°C under vacuum and freeze-dried. Antimicrobial activity was evaluated against eight common food pathogens using agar well diffusion assay as per method reported before[5] with some modifications. The method is based on the principle that involves the ability of fermented extracts to inhibit the growth of microorganisms, as exhibited by clear zones of inhibition. To check the antimicrobial activity, nutrient agar plates were used for bacterial strains and Potato Dextrose Agar (PDA) used for fungi and yeast strains allowed to solidify. Then the nutrient agar plates were inoculated with 100 µL of overnight active culture of indicator strains. The soft agar was allowed to solidify. Fungi and yeasts were maintained on potato dextrose agar (PDA) slopes and stored at 4ºC. Conidial spores were harvested in sterile distilled water containing approximately 10 conidia/ml. This conidial suspension was used for determining the antifungal activities of fermented extracts. The plates were refrigerated at 4°C for 1h before several wells were punched out of agar using sterile cork borers (10 mm diameter). The wells were then filled with 100 µL of fermented extracts. The plates were once again refrigerated at 4°C for 3-4 h to facilitate the diffusion of fermented extract and were incubated at 37°C for 24-48 h for bacteria, 3 and 5 days depending on the type of fungi at 25-28 ºC. The diameter of the zone of inhibition extending laterally around the well was measured and a clear zone of 1mm or more was considered positive inhibition.

5. Anticancer effect of wheat bran extraction

5.1 Hematoxylin and eosin staining of MG-63 cell line

Hematoxy; eneans Eionstrai was performed according to the reported paper[20] where IC50 treated and untreated cells were harvested by cold centrifugation (Jouan-Ki-22, France) residual adherent cells were trypsenized and processed in the same way. Deposited cells were re-suspended in 0.5 ml of phosphate buffer saline (PBS) Fifty microliters of treated and untreated cells were dispensed and spread in a circular way on clean slides (3 for each treatment).

Slides were air-dried, methanol fixed and rehydrated in descending concentrations of alcohol (100%, 90%, 75% and 50%). Slides were washed in distilled water for 5 min. The slides were immersed in filtered hematoxylin (HE) stain for 3 min and washed with distilled water twice. Slides were immersed in filtered eosin stain for 5 seconds and washed with distilled water. Dried slides were immersed in xylene, mounted with Canada balsam then coverslips were placed and left to dry. Ten microscopic fields of each slide were photomicrographed using the power of 400×. This was done using a digital camera (Canon, Japan), which was mounted on a light microscope. Images were transferred to the computer system for analysis. Field selection was based on the presence of the highest number of apoptotic cells. The photomicrographs were qualitatively evaluated for the presence of morphological criteria of apoptosis.

5.2 Cytotoxicity

Cytotoxic effect of different concentrations of wheat bran extraction was determined using 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, where growth medium was decanted from 96-well micro titer plates pre-cultured MCF-7 cells. Wheat bran extraction was applied in double fold serial dilutions to MCF-7 pre-cultured plates. Untreated wells served as negative control and plates were incubated at 37ºC for 24 h. Post incubation, the plates were washed three times with phosphate buffer saline (PBS) as 250 µl/well. Fifty µl of MTT solution (0.5 mg/ml) were added to each well and plates were incubated for further 4 h at 37ºC. Plates were PBS washed three times and the formed blue colored formazan was dissolved by adding 50 µl/well DMSO (Sigma Aldrich-USA) followed by shaking the plates for 10 mins at room temperature. Optical density (OD) was measured at 570 nm using ELISA plate reader. The percentage of cellular viability was calculated. The half maximal inhibitory concentration (IC_{50}) was determined as the concentration resulting in 50% inhibition of cellular growth following 24 h exposure to wheat bran extraction compared to the untreated control cells using Graph Pad prism software version 5 (S. Diego-USA)

5.3 Hematoxylin and eosin staining

Fifty micro liters of ELF-EMF treated MCF-7 either in presence or in absence of wheat bran extraction were dispensed on clean slides (3 slides for each treatment). Slides were air-dried, methanol fixed and rehydrated using descending concentrations of alcohol (100%, 90%, 75% and 50%). Slides were washed with distilled water for 5 mins. The slides were immersed in filtered hematoxylin stain for 3 mins and washed with distilled water twice followed by immersion in filtered eosin stain for 5 seconds and washed with distilled water. Dried slides were immersed in xylene followed by mounting with Canada balsam. The coverslips were added to each slide and left to air dry. Microscopic field's photomicrographs were evaluated for the presence of morphological features of apoptosis.

6. Statistical analysis

The studied traits analyzed by using SAS program.[21] Comparing the means for each trait done by using the revised LSD. Pearson correlation coefficient was calculated among studied traits in two years.[22] This work is important to assure that there is a lot of applications of organic and inorganic compounds which are useful in different fields.[23-47]

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