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BIOACTIVE COMPOUNDS OF FERMENTED SOYBEAN NATTO AS ANTIOXIDANT AND ANTIMICROBIAL AGENTS.

Mahmod, Marwa H.[a]; Ferial M. Abu-Salem[a]; M. H. El-Kalyoubi[b] and A. Y. Gibriel[b],

[a] Dept. of Food Tech., National Research Center.

[b] Dept. of Food Sci., Fac. Of Agric., An Shams Univ.

ABSTRACT

Soybean and its products became one of the most important functional foods due to its bioactive compounds (isoflavones and bioactive peptides), which have a greet health benefits to human being. In this study, it was prepared a fermented soybean natto with different treatments: drying natto with oven, solar, undervacuum, and extracting them with different solvents to check its antioxidant and antimicrobial activities. Results showed that, acetonitrile extract had the highest amount of peptides followed by methanol extract then water came in the last order. On the other hand, acetonitrile extract had the strongest antioxidant and antimicrobial activities, meanwhile water and methanol extracts had a close effect in general, but still methanol extract had the higher activities than water extract. Methanol extracted all of total isoflavones and some of bioactive peptides, acetonitrile extracted bioactive compounds and some of isoflavones. Upon correlation of these results, it was found that the highest antioxidant and antimicrobial activities excess in acetonitril extract. Therefore, it was assumed that the antioxidant and antimicrobial activities of natto came from both, isoflavones and bioactive peptides, but bioactive peptides had stronger activities.

Keywords: Antioxidant, Antimicrobial, Isoflavones, Natto, Bioactive peptide

INTRODUCTION

Consumption of foods containing significant amounts of antioxidants may help the human body to reduce oxidative damage related to ageing and diseases, such as atherosclerosis, cancer and cirrhosis. The use of synthetic antioxidants, such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA) have safety concerns, even in food preservation. Therefore, attention has been focused on the use of natural antioxidants for inhibition or protection from oxidative damage. Nowadays, there is an increasing consumer awareness and health-consciousness which results in pressure to avoid the use of synthetic additives. These facts bring about the need for research regarding the use of natural additives or alternative methods in order to extend shelf life and/or improve food safety. Such a solution, as regards prevention of lipid oxidation and microbial growth could be the use of natural antioxidants, as many of them also exhibit antimicrobial activity.

Soybean and its products are important sources of antioxidant compounds like polyphenols, including isoflavones (Fritz *et al*., 2003), and hence may be treated as a consummate functional food because of its innumerable desirable characteristics (Tripathi and Misra, 2005). Isoflavones are secondary plant metabolites, which constitute a group of natural

bioflavonoids synthesised almost exclusively by plants of Leguminaceae family (Fritsche and Steinhart, 1999). They occur in large amounts in soybeans and soy products, chickpeas, beans and other legumes, as well as clover, toothed medick and bluegrass (Reinli and Block, 1996). Isoflavones have similar structure to estrogen and can exhibit weak estrogenic effects and therefore belong to the so-called phytoestrogens. The principal compounds of this chemical group are daidzein and genistein. There is growing evidence that consumption of soybean and soy products might protect against hormone-dependent cancers, like breast and prostate cancer, and have beneficial effects with regard to cardiovascular diseases, osteoporosis and menopausal symptoms (Potter *et al*., 1998). In soybeans, isoflavones are present both as aglycones and as glycosides (β-glycosides, acetyl and malonyl glycosides), the latter being the predominant forms. The concentration and distribution of isoflavone forms in soybeans is influenced by the genotype, location and crop year, whereas in processed soy products it depends on the sort of soybean used as well as on the type of processing (Murphy *et al*., 2002). After consumption the glycosides are hydrolysed in the human gut to their aglycones, which are further metabolised, and excreted (Kulling *et al*., 2002). For this reason, several authors have preferred to determine the aglycones formed after acid (Mullner and Sontag, 2000) or enzymatic (Franke *et al*., 1994) hydrolysis. The antioxidant activity of soybean and soy products correlated well with total phenolic content (TPC) and total isoflavones (TI), whereas TPC showed higher correlation with TI (Akitha, 2009).

Soybean protein is the most extensively studied, especially the effects of temperature, pH and ionic strength on the functional properties of soy protein isolate/concentrate. Recently, functional properties of soy protein fractions, chemical and biochemical modified soy protein were reported. For instance, Jung *et al*., 2005 reported that low degree hydrolysis (2–4%) by endo-protease treatment of soy protein resulted in enhanced functional properties of soy flour. Hydrolysis of protein not only enhanced the functional properties but also generate bioactive peptides, which may have antioxidant activities. A wide range of peptides activities has been described, including antimicrobial properties, blood pressure-lowering (ACE inhibitory) effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of mineral absorption/bioavailability, cyto or immunomodulatory effects, and opioid activities. Moreover, some peptides are multifunctional and can exertmore than one of the effects mentioned (Meisel, 2004).

Large-scale bioactive peptides production is still a challenge, but fermentation can be a good source of bioactive peptides, new peptide sequences may also be generated during fermentation. Future clinical studies will clarify the physiological importance of soy peptides and their role in preventing chronic diseases.

"Natto" is a traditional fermented soybean food which is indigenous to Japan. Lipid in the natto is known to be resistant to oxidative deterioration by the action of antioxidants which might be produced during fermentation. In addition to antioxidant activity, isoflavonoids showed a significant inhibitory action against bacteria (Dastidar *et al*., 2004).

The present study aimed to prepare fermented soybean natto and study the effect of drying natto with oven, sun, undervacuum, and the bioactive compounds, isoflavones and peptides, the antioxidant and antimicrobial activities of these compounds was also evaluated.

MATERIALS AND METHODS

Materials:

Soybean *(Glycine max)* was obtained from The Agriculture Research Center-Giza-Egypt at 2007 season. The strain of *Bacillus natto* (NBRC 13169) which was obtained from National Institute of Technology and Evaluation Biotechnology Center (NITE), Japan. Genistin, genistein, dadzin and daidzein, which used as isoflavone standard, were obtained from sigma Chemicals Co.U.S.A.

Natto preparation: For Natto processing, the method of Wei *et al*., (2001) was applied as follows: Soybean was soaked in tap water at (1:3 w/v ratios) for 24hr to avoid any fermentative acidification. The soaked beans were cooked by autoclave unit at 121^{o}C for 3min. Fifty grams of cooked soybean was cooled to 38^{o} C and inculcated with *Bacillus* strain, and then incubated at 38^{o} C for 24 hrs. Natto product was obtained and stored until analysis.

Drying of natto: Natto was dryed using three methods: solar drying (60^{o}C), under vacuum drying (60^{o}C) and oven drying (60^{o}C).

Bioactive compounds extracts: the prepared samples were extracted by the following three methods:

i) Acetonitrile extract (peptide extraction): The samples were extracted 1 gm/ml: a mixture of water, acetonitrile and trifluoroacetic acids (TFA) (50:50:1 w/w), sonicated for 5 min, vortexed for 2 min, then centrifuged (Gibbs *et al*., 2004).

ii) Methanol extract (isoflavones extraction): One gram of samples were extracted with 12 ml of 80% methanol, filtrated and finally evaporated (Duke *et al*., 2003).

iii) Water extract: One gram samples of natto was mixed with 20 mL distilled water, homogenized and centrifuged (10000 rpm, 30 min), finally filtered (zhu *et al*., 2008).

Soybean Isoflavones Analysis.

Samples were analyzed by HPLC for their content of daidzin. genistein, genistin, daidzein according to the method described by Duke *et al*., (2003). The isoflavones were detected using a UV detector (Water 486) while on-line monitoring was done at 260 nm. Data were integrated and recorded using a Millenium chromatography manger software 2010 (Water Milford MAO1757).

Determination of peptide content

Peptide was determined according to the *o*-phthaldialdehyde method of (Zhu *et al*., 2008). Fifty microlitre of sample was added to 2 mL *o*-phthaldialdehyde (OPA) mixture [50 mL of a mixture containing 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% (w/w) sodium dodecyl sulfate (SDS), 40 mg of OPA dissolved in 1 mL methanol, 100 µl of β-mercaptoethanol, and 21.4 mL distilled water]. After a 2 min incubation at room temperature, the

absorbance at 340 nm was measured. The peptide content was calculated on the basis of the standard curve constructed using L-glutathione (reduced form) as a standard.

Determination of antioxidant activity:

The antioxidant activity of samples was determined using the modified method of (Zhu *et al.,* 2008). An aliquot (0.1 mL) of the DPPH radical solution (0.25 mM in ethanol) and 0.1 mL of the sample was added to the microplate and then the mixture was shaken vigorously for 20 min in the dark. The decrease in absorbance was measured at 520 nm against a blank (without sample). The DPPH radical scavenging activity of the sample was compared with that of a reference standard, BHA.

DPPH scavenging effect % = A_{blank}-A_{sample} /A_{blank} X 100

Where A = absorbance Blank = all reagents except sample

Screening of the antimicrobial activity

The antimicrobial activity was screened using disc diffusion method according to Zakaria *et al.,* (2007). The source of microorganism arterials in the following bacteria were tested: *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Bacillus cereous* (ATCC 10907), *Clostridium perfringens* (ATCC 13124) and *Staphylococcus aureus* (ATCC 29213) were obtained from NAMERO.

Statistical analysis

Data were subjected to statistical analysis using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1998).

RESULTS AND DISCUSSION

Soybeans (Glycine max) are considered to be a protein sources, complementing grain proteins, in Asian countries. In addition to protein, soybeans also contain various nutrients and functional components including isoflavonoids, which have strong antioxidant activities, anticancer, antilipodemic, antithrompotic, antihypertensive effect. Soybeans contain 0.1 to 5 mg total isoflavones per gram, primarily genistein, daidzein, and glycitein (Velasquez *et al*., 2007).

Fermentation of soybeans is an excellent processing method for improving nutritional and functional properties due to the increased content of small bioactive compounds. (Kwon *et al*., 2010). During preparation of fermented soy foods, 6-o-malonylglucosides, the most prevalent soybean isoflavones, are converted to aglycon form, which have the highest bioactive form (Murphy *et al*., 1999), also, fermentation produce peptides with bioactivities as antioxidant, anticancer, anti-inflammatory, angiotensen converting enzymes inhibitors (Inoue, *et al*., 2009), which make fermented soya an emerging area of research with great promise. (Mejia *et al*., 2009). Peptides from soy are currently the subject of investigation for new drugs and functional food ingredients for gut health and modulating the intestinal absorption of nutrients (Shimizu *et al*., 2007).

The present study, drying natto samples with oven, sun, undervacuum, is a trial trying to keep and improve its bioactive compounds, isoflavones and peptides, then extracting them with water, methanol and acetonitrile in order to check their bioactivities.

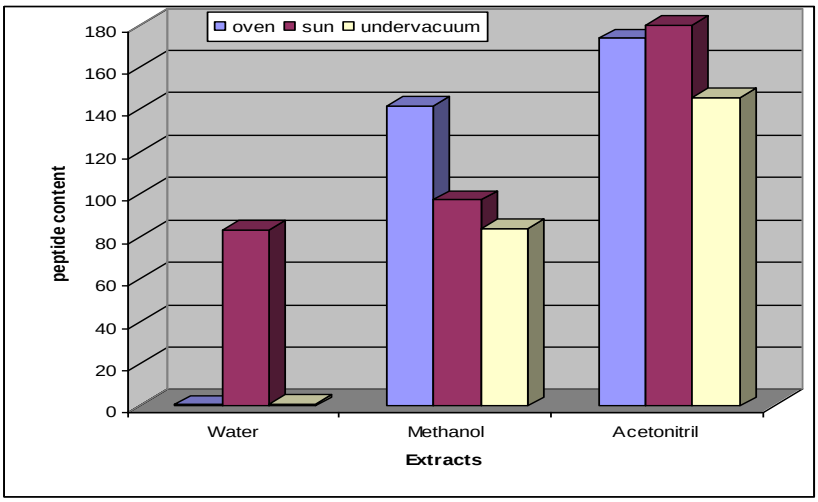

Fig (1): Effect of treatments on peptide content of natto.

Peptide content of Natto as affected by different treatments

Table (1) shows the effect of drying methods and extracting solvents on the peptide content of natto which revealed that the acetonitrile extract had the highest amount of peptides followed by methanol extract then water came in the last order. This may be due to the solubility and polarity of peptides depending on the peptide length, molecular weight and amino acids variety consist. The effect of drying method was found to be depending on the extracting solvent. The highest content of peptide was observed by extracting with acetonitrile using solar drying (Fig 1). This means that other methods of drying denaturating the proteins and peptides in natto during process. The results of water extract were in agreement with the results of (Zhu *et al*., 2008).

Table (1): Peptide content (ml/l) of Natto with deferent treatments.

solvent / Drying method	*Water*	*Methanol*	*Acetonitril*
Oven	0.63±0.06	141.78±0.29	173.45±0.20
Solar	83.28±0.20	97.24±0.20	179.43±0.15
Undervacuum	0.86±0.10	83.62±0.17	145.52±1.99

Isoflavones content of Natto as affected by treatments

The effect of extraction solvents on the total isoflavones content and distribution of isoflavones forms are shown in Table (2). It was clear that the methanol extract had the highest amount of total isoflavones, due to isoflavones polarities. Solar drying method had the highest amount of isoflavones compared to with different methods of drying, followed by undervacuum (Table 3); the decrease in isoflavones content could be related to heating and drying time.

Table (2): Effect of extracting methods on isoflavones of natto using solar drying

Isoflavones / Solvent	*Dadzin*	*Genistin*	*Dadzein*	*Genistein*	TOTAL
Acetonitril	5.02	1.455	5.536	0.8835	12.89
Methanol	48.8	9.562	89.5	17.52	149.16
Water	11.14	0	0	0	11.14

Table (3): Effect of drying methods on total isoflavones of natto

Solvent / Drying method	*Acetonitril*	*Methanol*	*Water*
Solar	12.89	149.16	11.14
Oven	68.23	52.28	6.35
Undervacuum	10.497	134	5.46

Fermentation did not cause a significant loss of isoflavones, but generated a different isoflavone distribution. Compared to the cooked soybean, the final product contained 6.5 times higher aglycons and 57% lower glucosides. Regarding to the glucosidic isoflavones content, Fig (2) showed the differences in total isoflavones between soybean flour and fermented soybean nattto. It was clear that the heating process (boiling) of soybean, which is a step of preparing natto, decrease the total isoflavones. Wang and Murphy, (1996) found the same effect of soybean processing steps on isoflavones content in fermented soybean tempeh; whereas each processing step led to losses in isoflavones. Cooking could increase the speed of leaching of isoflavones. However, cooking did not alter the distribution of isoflavones.

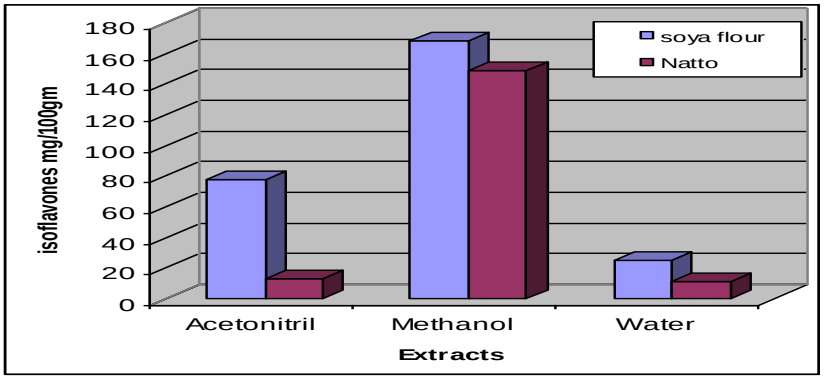

Fig (2): Total isoflavones content of soybean flour and fermented Natto

Antioxidant activity of soybean bioactive compounds

The antioxidant activity of natto came from both, isoflavones and bioactive peptides. Table (4) showe the antioxidant activities, IC_{50} and the

concentration in BHA ppm of bioactive compounds of fermented soybean natto in water, methanol and acetonitrile extracts.

Table (4): antioxidant activities, IC_{50} and the concentration in BHA ppm of bioactive compounds of fermented soybean Natto in water extrac

Extraction solven	Drying methods	*%Scavenging Activities*	*IC_{50}*	*concentration of antioxidant in ppm BHA*
methanol extract	**oven**	19.20±0.17	80.80±0.17	369.41
	solar	32.17±0.17	67.83±0.17	621.23
	undervacuum	28.55±0.25	71.45±0.25	550.89
Acetonitril extract	**oven**	57.07±0.04	42.93±0.04	1104.58
	solar	57.75±0.02	42.25±0.02	1117.76
	undervacuum	57.14±0.04	42.86±0.04	1105.97
water extract	**oven**	10.23±0.21	89.77±0.21	195.18
	solar	69.78±0.39	30.22±0.39	1351.34
	undervacuum	23.69±0.04	76.31±0.04	456.60

IC_{50}: inhibition concentration of 50% of DPPH

Results showe that the highest antioxidant activities were for acetonitrile extract which contain all of bioactive peptides and some of isoflavones. From Figs (1,3,4) it could be stated that there was a correlation between peptide content and antioxidant activities more than that between the isoflavones content and antioxidant, which mean that the antioxidant activities of peptides is more higher than isoflavones.

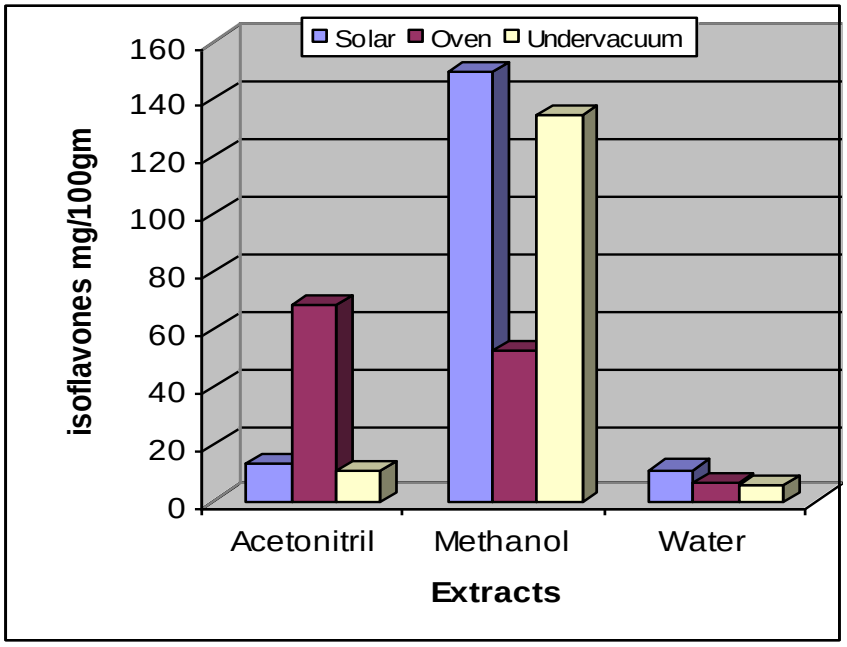

Fig (3): Effect of drying methods on total isoflavones content of Natto

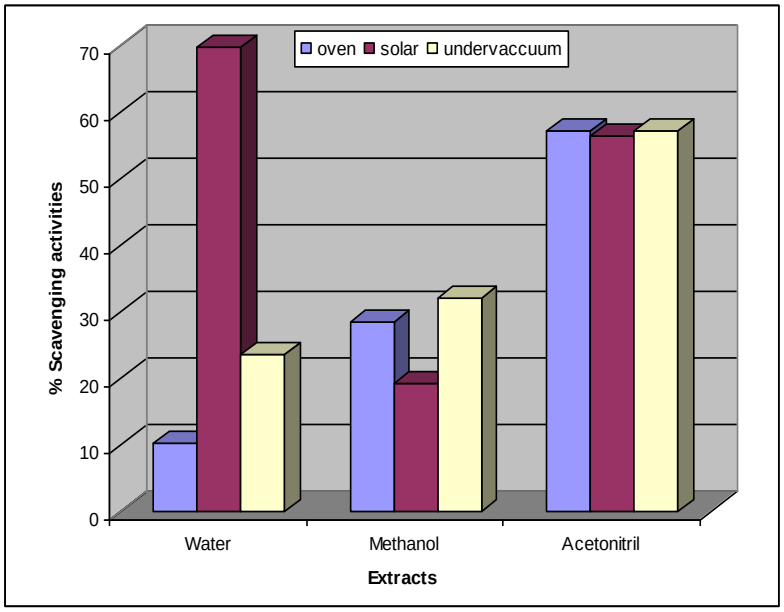

Fig (4): Effect of extraction methods on the antioxidant activities of natto

Antimicrobial activities of soybean bioactive compounds

Non-polar components such as phenolic, responsible for the antimicrobial properties of phenolic compounds, are generally considered effective only against Gram-positive bacteria (Ferna´ndez-Lopez *et al*., 2005). This could be explained by the structural differences of the bacterial cell wall of Gram-positive and Gram-negative bacteria. Gram-negative bacteria, apart from the cell membrane, possess an additional layer, the outer membrane, which consists of phospholipids, proteins and lipopolysaccharides, and this membrane is impermeable to most molecules (Georgantelis *et al*., 2007). Nevertheless, the presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da (Abee, *et al*., 1995).

Figs (5,6) showe the antimicrobial activities of natto extracts against *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Bacillus cereous* and *Clostridium perfringens* respectively. It was clear that the acetonitrile extract have the stronger antimicrobial activities than other extracts. Water and methanol extracts have a close effect in general, but still methanol extract have highest antimicrobial activity than that of water extract. This means that both of isoflavones and bioactive peptides are sheared in the antimicrobial activities of soybean Natto.

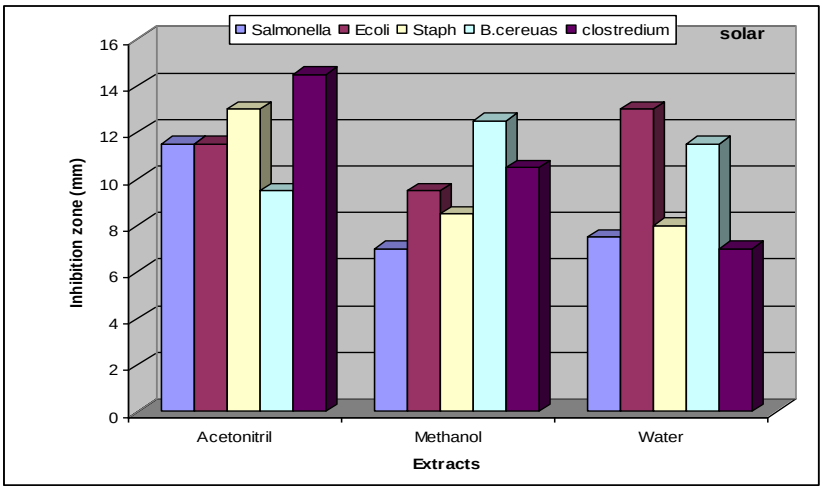

Fig (5): Antimicrobial activities of Natto extracts on pathogenic bacteria.

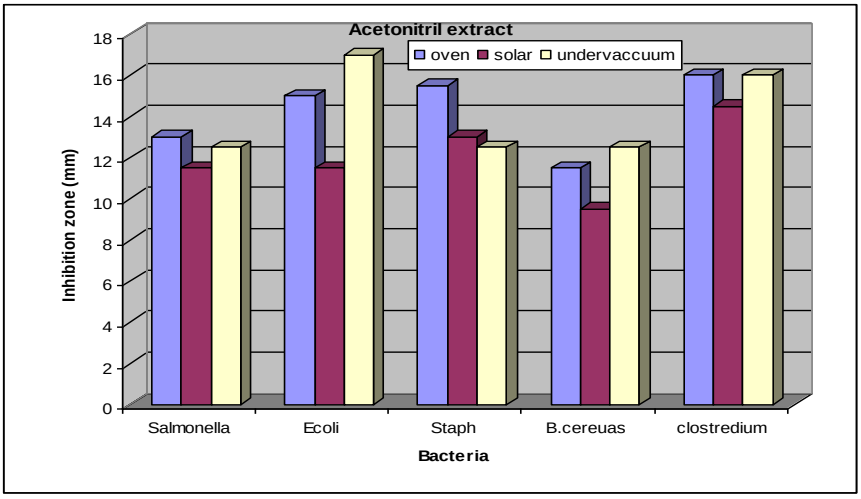

Fig (6): Antimicrobial activities of Natto extracts on pathogenic bacteria

Some of phenolics are organic molecules with high molecular weight bulky substitute, which could reduce their ability to reach the bacterial cell membrane of Gram-negative bacteria. However, growth inhibition of Gram-negative bacteria has been reported, especially in combination with factors that can disturb cell membrane integrity and/or permeability, such as low pH values and increased NaCl concentrations (Del Campo *et al*., 2000). Helander *et al*., (2001) found a similar effect which was arisen from the combination of rosemary with chitosan, since the latter is able to interact with membranes and cell wall components. From the above mentioned studies, it

could explained why it had antimicrobial activities against both grams positive and negative, as a result of peptide and some other components excess together with isoflavones, which may increase the ability of isoflavones to cross the cellular membrane and destroy its permeability which lead to killing the microbes.

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المركبات ذات النشاط الحيوى لفول الصويا المتخمر ناتو كعوامل مضادة للاكسدة ومضادة للميكروبات

مروة حنفى محمود*، فريال محمد ابوسالم*، ممدوح حلمى القليوبى و احمد يوسف جبريل****

*** قسم الصناعات الغذائية – شعبة الصناعات الغذائية والتغذية – المركز القومى للبحوث**

**** قسم علوم الاغذية – كلية الزراعة – جامعة عين شمس**

أصبح فول الصويا ومنتجاته واحد من الأغذية الوظيفية الأكثر أهمية ويرجع ذلك الى احتوائه على مركبات نشطة حيويا (الايسوفلافون والببتيدات النشطة حيويا) التي لها فوائد صحية عديدة للإنسان. فى هذه الدراسة، تم عمل منتج الصويا المتخمر الناتو واجريت له معاملات مختلفه للتجفيف مثل التجفيف فى الفرن والتجفيف الشمسى والتجفيف تحت ضغط، كذلك تم استخدام مذيبات مختلفة للاستخلاص وذلك لدراسة النشاط المضاد للاكسدة والمضاد للميكروبات. وأظهرت النتائج أن مستخلص الاسيتونيتريل احتوى على أكبر قدر من البتيدات يليه المستخلص الميثانولى، ويأتى المستخلص المائى فى المرتبة الاخيرة. وعلى الجانب الاخر فان مستخلص الاسيتونيترل قد اظهر أعلى نشاط مضاد للاكسدة ومضاد للميكروبات، وقد تقاربت نتائج كلا من المستخلص الميثانولى والمستخلص المائى كمضادات اكسدة ومضادات ميكروبية ولكن مازال المستخلص الميثانولى محتفظ بنشاط اعلى من المستخلص المائى. ونجد ان الميثانول قد استخلص جميع الايسوفلافونات الموجودة وبعض البتيدات النشطة حيويا بينما الاسيتونيتريل استخلص البتيدات النشطة حيويا كلها وبعض الايسوفلافونات. وعند ربط العلاقة بين هذه النتائج وجد ان أعلى نشاط مضاد للأكسدة ومضاد للميكروبات كان لمستخلص الاسيتونيتريل. وعلى ذلك ، يفترض أن الأنشطة المضادة للأكسدة والمضادة للميكروبات من الناتو جاء من كلا من البتيدات النشطة حيويا والايسوفلافون، ولكن البتيدات النشطة حيويا قد كان لها تاثير اعلى.

قام بتحكيم البحث

أ.د / أحمد عبد العزيز الرفاعى — كلية الزراعة – جامعة المنصورة

أ.د / هيام محمد أحمد ابراهيم — المركز القومى للبحوث