Volume 37 – No. 2 - 2015
pp. 43 - 84

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AFS – Advances in Food Sciences
Continuation of CMTL founded by F. Drawert

Production by PSP – Parlar Scientific Publications, Angerstr. 12, 85354 Freising, Germany
in cooperation with Lehrstuhl für Chemisch-Technische Analyse und Lebensmitteltechnologie,
Technische Universität München, 85350 Freising - Weihenstephan, Germany

Printed in GERMANY – ISSN 1431-7737

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ORIGINAL PAPERS

MINI-REVIEW

PRODUCTION OF HIGH DIETARY FIBER BREAD FORTIFIED WITH QUINOA FLOUR

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ABSTRACT

The present work was conducted to study producing of high fiber bread with good nutritional value and good sensory properties from wheat flour (WF) (72% extraction rate) and wheat bran (WB) at a ratio of 2:1 as control high fiber bread. Then, WF was replaced with quinoa flour (QF) at levels from 10-50%. Chemical composition, sensory evaluation, biological value and chemical score of the high fiber breads were evaluated. The results showed that organoleptic characteristics of high fiber bread from WF with 10-30% QF replacement gave good values of color, taste, odor, softness, chewiness and overall acceptability compared with control breads.

Results also showed that addition of QF caused an increase in protein, ether extract, crude fiber, ash and total dietary fiber when compared with control high fiber bread. In addition, QF substitution increased the biological value with regard to control bread (74.40 and 58.93%), respectively. Besides high scores in chemical composition, QF-substituted high fiber breads were high for all essential amino acids, especially lysine, threonine, and tyrosine + phenylalanine. This indicated that QF could be well incorporated in high fiber breads with good organoleptic characteristics and nutritional values when compared to high fiber bread controls.

KEYWORDS: High fiber bread- quinoa flour- biological value, good organoleptic characteristics

1. INTRODUCTION

Today, health-conscious consumers are looking for foods that will fit their health lifestyle. Bread is one of the most consumed foods and has a critical position in consumer foods because of its nutritional aspects, energy providing and food habits. In recent years, application of additives and different ingredients in order to enhance quality and nutritional value of bread has been of interest and to date various ingredients have been added to it.

Sidhu *et al.* [1] studied the effect of the type of miller`s bran and levels of addition. Particle size and addition of wheat germ on the chemical composition of high fiber toast bread was investigated. The bran and germ fractions were found to be high in ash, protein, fat, and total dietary fiber contents. The wheat germ had a protein content of 27.88% compared with 11.35, 12.69 and 15.76% for white flour, whole wheat flour and red fine bran, respectively. The chemical composition of high fiber bread in terms of minerals, protein, fat and dietary fiber contents was found to be superior with regard to whole wheat flour (control) bread.

Dietary fiber (DF) is the food fraction that is not enzymatically degraded within the human alimentary digestive tract. The main components are cellulose and lignin, but also the hemicelluloses, pectin, gums and other carbohydrates are not digestible by the human digestive tract [2]. The DF fraction was increased due to beneficial effects on the reduction of cholesterol levels and the risk of colon cancer [3]. Fiber–rich breads obtained were also considered to be acceptable by the sensory panel. The DF composition of the final products revealed that these fibers are good for use as fiber–enriching agents in bread making.

Total DF content of whole wheat flour is 10.2% compared with 2.5% for white flour. On the other hand, the values for total DF in wheat bran range between 40 and 44%, thus making it an ideal natural supplement for producing high-fiber baked products [4].

Quinoa (*Chenopodium quinoa* Wild) is considered as a pseudo-cereal or pseudo-grain, but has been recognized as a complete food due to its protein quality. It has remarkable nutritional properties, not only from its protein content (15%) but also from its great amino acid balance. It is an important source of minerals and vitamins, and has also been found to contain compounds like polyphenols, phytosterols, and flavonoids. Quinoa has a high nutritional value and has recently been used as a novel functional food because of all these properties; it is a promising alternative cultivar [5]. Relative to cereal grains, quinoa proteins (QPs) are particularly high in lysine, the limiting amino acid in most cereal grains. Their essential amino acid balance is excellent and of a wider amino acid range than wheat bran. Quinoa was selected because of its natural wheat flavor, and

also being a good source of proteins as well as a rich source of B-complex vitamins and minerals [6].

According to the National Academy of Sciences [7], the magnesium, manganese, copper and iron present in 100 g of quinoa seeds cover the daily needs of infants and adults, while the phosphorus and zinc contents in 100 g are sufficient for children, but cover only 40-60% of the daily needs of adults. The potassium content can contribute 18-22% of infant and adult requirements, while the calcium content can contribute 10% of requirements, with higher lysine (5.1-6.4%) and methionine (0.4-1%) rates.

Wafaa and Shams [8] studied the benefits of quinoa seeds (*Chenopodium quinoa*, variety Titicaca) which are newly introduced in Egypt. Quinoa seeds were analyzed to evaluate their proximate composition. Moisture, protein, ether extract, crude fiber and ash contents of quinoa flour (QF) were 9.61, 13.97, 3.93, 10.31 and 3.67 % (dry basis), respectively. In addition, quinoa seeds had higher contents of most essential amino acids, especially lysine, than wheat flours. The incorporation of quinoa flour in balady bread manufacturing at different levels (10, 20, 30, 40 and 50 %) was concerned. The addition of 10 or 20 % quinoa flour did not substantially change rheological properties of the dough while the sensory characteristics of balady breads were accepted at 20 % supplementation level. It is important to note that the addition of quinoa flour to wheat flour could produce bread with a good nutritive value.

The objectives of this study were to produce high dietary fiber breads with good nutritional values and sensory properties by using wheat bran and quinoa flour.

2. MATERIAL AND METHODS

2.1 Materials

Wheat flour (72% extraction rate), Gemiza-9 variety, and wheat bran (that could pass through a 25-mesh screen) were obtained from the Crops Research Institute, Agricultural Research Center.

Quinoa (*Chenopodium quinoa*, variety Titicaca) seeds were obtained from the Crop Intensification Research Section Field, Crops Research Institute, Agricultural Research Center.

Other ingredients, such as dry yeast, improvers and salt, were purchased from local market.

2.2 Methods

2.2.1 Preparation of Quinoa flour (QF)

Quinoa flour was prepared according to Miranda *et al.* [9]. Seeds of quinoa (*Chenopodium quinoa*) were washed with water at 60 ºC (with agitation) for 1 h, with a seeds to water ratio of 1:10 (w/w). Then, drying was carried out at 60 ºC using a convective dryer; and then, it was ground in a cyclone sample mill into flour that could pass through a 60-80 mesh screen.

2.2.2 Baking and organoleptical properties

High fiber bread blends were prepared by partial replacement of wheat flour (WF 72% ext. rate) with 30% wheat bran (as control). Then, balady bread blends were prepared by partial replacement of WF (72% ext. rate) with different levels (10, 20, 30, 40 and 50%) of quinoa flour (QF). High fiber bread loaves were organoleptically evaluated by 12 panelists.

2.3 Analytical methods

2.3.1 Chemical composition of samples

Moisture, crude protein, crude fat, ash and crude fiber of wheat flour (72% extraction rate), wheat bran and quinoa flour, and their different blends were determined according to A.O.A.C. [10].

2.3.2 Determination of minerals and amino acids content

Mineral contents of WF (72% ext.), wheat bran and quinoa flour, and their different blends (magnesium, sodium, zinc, manganese, iron, calcium, potassium and copper) were determined using a PyeUnicamSp 1900 atomic absorption spectrophotometer after dry ashing according to the method described in A.O.A.C. [11]. Amino acid contents of WF, WB, QF, and their different blends were determined according to the method described by Pellet and Young [12].

2.3.3 Determination of total dietary fiber (TDF)

Total dietary fiber (TDF) was determined in the studied samples and the produced high fiber breads according to A.O.A.C. method [13].

TABLE 1 - Formulaof different high dietaryfiberbread blends (100g).

Ingredients	(1) Control	(2)	(3)	(4)	(5)	(6)
Wheat flour	67	60.3	53.6	46.9	40.9	33.5
Wheat bran	33	33	33	33	33	33
Quinoa flour	-	6.7	13.4	20.1	26.8	33.5
Yeast	5	5	5	5	5	5
Improvers	3	3	3	3	3	3
Salt	2	2	2	2	2	2

2.3.4 Chemical score and biological value

Chemical score of essential amino acids (EAA) was relatively calculated, according to FAO/WHO [14], by using the following equation:

Chemical score (%) = EAA in crude protein x 100/EAA of FAO/WHO

2.3.5 Biological value

Biological value (positively correlated with lysine concentration) was calculated as follows (Eggum *et al.* [15]):

Biological value (%) = 39.55+8.89 x lysine (g/100g protein)

2.4 Statistical analysis

Statistical analysis of the resulting data was carried out according to Fisher [16]. LSD (least square difference) test was used to compare the significant differences between means of Waller-Duncan treatment [17].

3. RESULTS AND DISCUSSION

3.1 Chemical composition of raw materials

Chemical compositions of raw materials (WF 72% ext., WB and QF) are presented in Table 2. Results revealed that WB contained higher amounts of protein, ether extract, crude fiber, ash and total dietary fiber (14.68, 3.71, 10.84, 3.80 and 39.11%, respectively), followed by QF (13.79, 3.59, 10.23, 3.58 and 11.12%), compared with WF (10.31, 1.26, 1.60, 0.97 and 2.14%, respectively. Results are in agreement with Sidhu *et al.* [1]. Meanwhile, WF contained a higher amount of carbohydrates (85.86%) than WB and QF (66.97 and 68.81%).

3.2 Mineral contents

Table 3 shows mineral contents of WF, WB and QF. Data revealed that K, Mg and Mn were higher in WB than WF and QF. QF contained higher amounts of Na, Ca and Fe than WF or WB. These results are in agreement with those obtained by Bharagava *et al.* [18] and Hareedy *et al.* [19] who mentioned that quinoa grains contain large amounts of minerals like Ca, Fe, Zn, Cu and Mn, and Ca and Fe were significantly higher than in most commonly used cereals.

3.3 Amino acid contents

Amino acid contents in WF, WB and QF are shown in Table 4. WB contained higher amounts of essential amino-acids, except for lysine and tyrosine, which were higher in QF. In addition, WB contained higher amounts of all non-essential amino acids, except for aspartic acid and arginine, which were higher in QF. These results are in agreement with those obtained by Hareedy *et al.* [19]. Moreover, Abugoch James [20] reported that quinoa proteins have higher histidine content than barley, soy or wheat protein. Koziol [21] found that quinoa is rich in histidine and lysine (3.20 and 6.10% of protein composition, respectively). Also, Abugoch *et al.* [22] reported that amino acid analysis showed that

TABLE 2 - Chemical composition of raw materials (on dry weight bass%).

Ingredients	Protein	Ether extract	Crude fiber	Ash	Carbohydrates	Total Dietary fiber
Wheat flour	10.31	1.26	1.60	0.97	85.86	2.14
Wheat bran	14.68	3.71	10.84	3.80	66.97	39.11
Quinoa flour	13.79	3.59	10.23	3.58	68.81	11.12

TABLE 3 - Mineral contents of different raw materials (ppm).

Ingredients	Na	K	Mg	Ca	Mn	Fe
Wheat flour	94.32	84.61	22.10	26.50	8.16	18.9
Wheat bran	98.7	220.32	140.36	78.46	20.60	23.8
Quinoa flour	154.11	180.30	57.91	82.13	18.20	151.30

TABLE 4 - Amino acid composition of raw materials (g/100 g protein).

Essential amino acids	Wheat flour	Wheat bran	Quinoa flour	Non-essential amino acids	Wheat flour	Wheat bran	Quinoa flour
Lysine	1.8	2.89	4.61	**Glutamic**	30.41	32.95	13.22
Methionine + cystine	3.24	5.25	3.39	**Aspartic**	3.65	6.40	6.81
Isoleucine	2.95	3.42	2.85	**Proline**	13.12	10.32	4.24
Leucine	5.82	7.11	5.01	**Alanine**	3.53	4.86	2.93
Phenylalanine	4.42	4.95	3.12	**Glycine**	4.39	4.92	4.34
Tyrosine	2.39	2.58	2.82	**Serine**	4.51	4.43	3.17
Threonine	2.18	3.75	2.73	**Arginine**	3.52	7.12	9.20
Valine	3.15	4.92	4.08	**Histidine**	1.81	3.98	3.12
Total essential amino acids	25.95	34.87	28.61	**Total non-essential amino acids**	64.94	94.98	47.03

quinoa is an excellent source of lysine, methionine, or cystine, in addition to other essential amino acids, and it meets or exceeds the recommendation for proper amino acid nutrition.

3.4 Sensory evaluation of high fiber bread

Organoleptic characteristics of high-fiber bread loaves produced from WF (72% ext. ratio) mixed with WB at ratio 2:1 as control sample of high fiber bread (No. 1) and WF replaced with quinoa flour at 10, 20, 30, 40, and 50% (No. 2-6) are shown in Table 5). High-fiber bread produced from WF and 10-30% replacement by quinoa flour gave good values of color, taste, odor, softness, chewiness, and overall acceptability. Results also indicate that high fiber bread produced from blends 40 and 50% of QF caused a decrease in the same previous parameters compared to control. These results are in agreement with Lopes Almeida *et al.* [23] who studied the effects of adding different dietary fiber sources (wheat bran, resistant starch and locust bean gum), on process and quality parameters of pan bread, and reported that WB additions above 10 g/100 g flour yielded good results in the sensory evaluation of crumb colour and appearance. Breads with high WB, LBG and RS contents obtained high positive purchase intention percentages. The acceptance of crust colour, crust appearance, aroma and taste was not affected by the addition of the different dietary fibers, within the concentration ranges studied. The above results showed that quinoa flour could be incorporated in producing of high-fiber bread with good organoleptic characteristics at 30% of replacement.

3.5 Chemical composition of control and quinoa-substituted breads

The nutritional quality of products depends on the quantity of product and quality of the nutrients. Therefore, chemical composition of control and quinoa-substituted bread (at 30% replacement; samples 1 and 4) are shown in Tables 6, 7 and 8. Results in Table 6 show that addition of QF caused an increase in protein, ether extract, crude fiber, ash and total dietary fiber compared with control bread (72% ext. WF and WB ratio 2:1). Data in Table 7 reveal that quinoa-substituted bread shows an increase in some mineral contents, such as Na, Ca and Fe, and causes a decrease in K, Mg and Mn contents.

Amino acid compositions of control and quinoa-substituted breads are shown in Table 8. It could noticed that addition of QF to WF (at 30% replacement) caused increase in all essential amino acids, except leucine. Meanwhile, QF caused a decrease in some non-essential amino acids like glutamice, proline and serine.

3.6 Effect of quinoa flour substitution on biological value and chemical score

Biological value and chemical score of QF-substituted high-fiber bread and control sample are shown in Table 9. Results indicate that QF increased the biological value compared with control high-fiber bread (74.40 and 58.93%, respectively). Besides, chemical score of quinoa-substituted bread was higher for all essential amino acids, especially lysine, threonine and tyrosine + phenylalanine).

TABLE 5 - Organoleptic characteristics of bran bread and different blends.

Blends	Colour	Taste	Odour	Softness	Chewiness	Overall acceptability
1-	19.10 ± 0.73a	18.30±1.05a	19.40±0.84a	18.25±1.16a	18.13±1.46a	93.13±5.06a
2-	18.60±1.07ab	18.30±1.33a	18.80±1.35ab	17.75±1.16ab	17.63±1.30ab	91.00±5.48ab
3-	18.50±0.85ab	18.20±1.13a	19.20±0.91a	17.13±1.35abc	17.13±1.36abc	89.63±4.84ab
4-	18.40±0.84ab	16.50±0.56b	18.40±1.64abc	16.75±0.89bc	16.50±1.31bcd	86.38±3.89bc
5-	17.90±1.01b	16.90±1.28b	17.60±1.57bc	16.38±1.19c	15.75±1.49cd	84.13±4.03cd
6-	16.00±1.15c	17.00±1.56b	17.10±2.46c	16.00±1.07c	15.13±1.13d	80.00±5.13d
LSD at 5%	**0.8722**	**1.0748**	**1.4087**	**1.1573**	**1.3574**	**4.813**

No.1 (control): High fiber bread loaves produced from wheat flour (72% ext. ratio) mixed with wheat bran at ratio (2:1). No. 2, 3, 4, 5 and 6: High-fiber bread (wheat flour substituted with quinoa flour at 10, 20, 30, 40 and 50%, respectively).

TABLE 6 - Chemical composition of control and quinoa-substituted breads.

Samples	Protein	Ether extract	Crude fiber	Ash	Carbohydrates	Total dietary fiber
Control	12.38	3.82	5.11	2.94	75.75	18.12
Q. S. b	13.22	4.53	6.92	4.48	70.85	21.46

Control =high-fiber bread loaves produced from wheat flour (72% ext. ratio) mixed with wheat bran at ratio (2:1); Q. S. b = high-fiber bread (wheat flour substituted with quinoa flour at 30%).

TABLE 7 - Mineral content of control compared with quinoa-substituted bread (ppm).

Samples	Fe	Na	K	Mg	Ca	Mn
Control	20.85	985.41	135.48	64.23	45.18	13.30
Q. S. b	54.12	1128.80	101.76	29.30	57.11	9.45

Control = high-fiber bread loaves produced from wheat flour (72% ext. ratio) mixed with wheat bran at ratio (2:1); Q. S. b = high-fiber bread (wheat flour substituted with quinoa flour at 30%).

TABLE 8 - Amino acids composition of control compared with highly substituted bread (g/100 g protein).

Essential amino acids	Control	Q. S. b	Non essential amino acids	Control	Q. S. b
Lysine	2.18	3.92	**Glutamic**	31.81	30.11
Methionine + cystine	4.36	4.98	**Aspartic**	7.29	8.78
Isoleucine	3.16	3.81	**Proline**	15.90	14.80
Leucine	4.76	4.61	**Alanine**	5.95	6.11
Phenylalanine	4.65	5.52	**Glycine**	3.87	6.92
Tyrosine	2.50	3.45	**Serine**	6.14	3.40
Threonine	2.78	3.65	**Arginine**	4.95	7.12
Valine	3.86	4.82	**Histidine**	2.78	3.24
Total essential amino acids	28.25	34.76	**Total non-essential amino acids**	23.69	80.48

Control = high-fiber bread loaves produced from wheat flour (72% ext. ratio) mixed with wheat bran at ratio (2:1); Q. S. b = high-fiber bread (wheat flour substituted with quinoa flour at 30%).

TABLE 9 - Effect of quinoa flour substitution on biological value and chemical score.

Essential amino acids	E.A.A. for control	E.A.A. for Q. S. b	FAO/WHO	Chemical score for control	Chemical score for Q. S. b
Lysine	2.18	3.92	5.50	39.64	71.27
Threonine	2.50	3.45	4.00	62.5	86.25
Methionine+Cystin	4.36	4.98	3.50	124.57	142.29
Valine	3.86	4.82	5.00	77.20	96.40
Isoleucine	3.16	3.81	4.00	79.00	95.25
Leucine	4.76	4.61	7.00	68.00	65.86
Tyrosine + phenylalamime	7.15	8.97	7.00	102.14	128.14
Biological value (%)	58.93	74.40	-	-	-

Control =high-fiber bread loaves produced from wheat flour (72% ext. ratio) mixed with wheat bran at ratio (2:1);
Q. S. b =high-fiber bread (wheat flour substituted with quinoa flour at 30%).

The authors have declared no conflict of interest.

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Received: September 17, 2014
Accepted: November 18, 2014

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INTERACTION OF PLANTAIN (*MUSA PARADISIACA*) PEEL EXTRACTS (UNRIPE, RIPE AND OVER RIPE) WITH KEY ENZYMES LINKED TO HYPERTENSION (ANGIOTENSIN-I CONVERTING ENZYME) AND THEIR ANTIOXIDANT ACTIVITIES (*IN VITRO*): A NUTRACEUTICAL APPROACH

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ABSTRACT

The unripe plantain pulp has been used in folklore medicine in the management of diabetes and hypertension. However, the peels which contribute about 40% of the plantain fruit weight may constitute serious environmental waste problem. Therefore, this study sought to investigate the antihypertensive and antioxidant properties of aqueous extracts of three different plantain peels in order to estimate their nutraceutical potentials. Aqueous extracts (1:100 w/v or 0.01 g/ml) of the plantain peels (ripe, unripe and over-ripe) were prepared, and their interaction with key enzyme enzyme linked to hypertension (Angiotensin-I converting enzyme) was assessed. Furthermore, the abilities of the extracts to scavenge 1,1-diphenyl-1-picryl-hydrazyl (DPPH) radical and prevent lipid peroxidation induced by $Fe^{2+}$and sodium nitroprusside (SNP) in rat hearts using thiobarbituric reactive species (TBARS) were subsequently determined. The results revealed that the unripe plantain peel (UPP) extract (IC_{50} = 14.93 µg/ml) had stronger inhibition of ACE activity than the ripe (RPP) and over ripe plantain peel (OPP) extracts. In a similar manner, UPP extract had a higher DPPH radical scavenging ability (IC_{50} = 218.43 µg/ml) than both RPP (IC_{50} = 230.41 µg/mL) and OPP (IC_{50} = 253.90 µg/mL) extracts. Also, incubation of the rat's heart in the presence of Fe^{2+} and SNP caused a significant increase ($P<0.05$) in the malondialdehyde (MDA) content. However, introduction of the plantain peel extracts caused a dose-dependent decrease in the MDA content, with the UPP extract having the highest inhibitory effect. The phenolic characterization of the peels using gas chromatography revealed the presence of astragalin, isoquercetin, *p*-coumaric acid, quercetin, rutin, catechin, protocatechuic acid, and cavarol in abundance. The result from this study may suggest that the inhibition of ACE activity, prevention of lipid peroxidation in the heart, and radical scavenging abilities of the peels may be a part of the possible mechanism through which the plantain peels exert their antioxidant and antihypertensive effects.

KEYWORDS: Plantain peels, polyphenols, antioxidants, malondialdehyde, antihypertensive properties, oxidative stress

1. INTRODUCTION

Hypertension is a physiological state in which the blood pressure is raised [1]. Persistent hypertension has been linked to several cardiovascular diseases (CVD), such as stroke, heart attack and heart failure [2]. Also, inflammation of the heart resulting from oxidative stress is regarded as the major cause of CVD [3]. Renin-angiotensin system plays a crucial role in the regulation of blood pressure, salt and water balance, and in the pathophysiology of cardiovascular diseases, such as congestive heart failure and hypertension [4]. Renin produces angiotensin-I from angiotensinogen and, thereafter, angiotensin-I converting enzyme (ACE) converts angiotensin-I to a potent vasoconstrictor, angiotensin-II [5]. Inhibition of ACE is considered to be a useful therapeutic approach in the management/treatment of high blood pressure in both diabetic and non-diabetic patients. Several drugs, such as captopril, lisinopril, enalpril and zofenopril, have been designed in this manner [6, 7].

Evidence has also shown that these damaging events in biological systems are caused by free radicals [8]. Fe is also a physiologically necessary components of many enzymes and proteins, and free Fe in the cytosol and mitochondria could cause considerable oxidative damage by acting catalytically in the production of ROS which has the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates resulting in a wide range of impairment in cellular function and integrity [9].

Polyphenols are common constituents of the human diet, present in most foods and beverages of plant origin. They are considered to contribute to the prevention of various degenerative diseases, such as diabetes and hypertension. This assumption originally came from *in vitro* studies, showing the antioxidant properties of several polyphenols and their ability to modulate the activity of various enzymes [10-12].

Plantain peels (40% of the plantain weight) are known to constitute a menace to the society, thereby adding to the worse problem of environmental pollution, particularly in places where ruminants (sheep and goat) are not allowed to roam about. Recent research revealed that fruit peels and seeds, such as grape seeds and peels [13], pomegranate peel [14], wampee peel [15] and mango seed kernels [16] may potentially possess antioxidant properties. The search for natural antioxidants from other parts of fruits and vegetables is widespread, since these other parts, such as seeds or peels are thrown away after the pulp has been used up. The question bearing on mind is why to throw it away when it can be used as a nutraceutical - with its chemotherapeutic potentials. Although plantains have been reportedly used in folk medicine for the management/prevention of hypertension, there is a dearth of information on whether the peels (unripe, ripe, and over-ripe, 40% of the total weight) constituting a waste problem may exert anti-hypertensive property. Hence, the objective of this study is to investigate the inhibitory effect of plantain peels (*Musa paradisiaca*) on angiotensin-I converting enzyme (key enzyme linked to hypertension) activity and Fe^{2+}-induced oxidative stress in rat heart – *in vitro* - to provide some possible mechanism by which they exert their anti-hypertensive property and can be used as nutraceuticals in pharmaceutical and food industry.

2. MATERIALS AND METHODS

2.1 Sample collection

Fresh mature unripe (UPP), ripe (RPP) and over ripe (OPP) plantain peels were collected from the Oja-Oba market in Akure, Ondo State, Nigeria. Authentication of these peels was carried out at the Department of Biology, Federal University of Technology, Akure, Nigeria.

2.2 Chemicals and reagents

Chemicals and reagents used, such as hippuryl-histidyl leucine substrate, gallic acid, or Folin-Ciocalteau's reagent, were procured from Sigma-Aldrich, Inc. (St Louis, MO); trichloroacetic acid (TCA) was sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany); acetic acid and $FeCl_3$ were sourced from BDH Chemicals Ltd. (Poole, England): Sodium carbonate, $AlCl_3$, potassium acetate, Tris-HCl buffer, sodium dodecyl sulphate, $FeSO_4$, sodium nitroprusside, potassium ferricyanide and ferric chloride were of analytical grade while the water was glass-distilled.

2.3 Sample Preparation

The plantain peels were grouped as collected, (unripe green peel, ripe yellow peel and over ripe brown peel). Each group was cleaned and rinsed with distilled water, and manually sliced thinly (0.5-1.0 inch thick). The sliced part was immediately sun-dried; the dried products were pulverized and passed through a 100-mesh sieve, producing a free-flowing powder.

2.4 Aqueous extraction

1 g of each sample was soaked in 100 ml of distilled water for about 24 h. The mixture was filtered and air-dried. The air-dried samples were kept at –20 ºC until usage for further analysis. All analyses were performed in triplicate, and results were averaged.

2.5 ACE inhibition assay

Appropriate dilutions of the extracts (0-200 µl) and 50 µl ACE (EC 3.4.15.1) solution (4 mU/ml) were incubated at 37 °C for 15 min. After pre-incubation, the enzymatic reaction was initiated by adding 150 µl of 8.33 mMhippuryl-histidyl leucine (Bz-Gly-His-Leu) in 125 mMTris-HCl buffer (pH 8.3) to the mixture and incubating at 37 °C for 30 min. After incubation, the reaction was stopped by adding 250 µl of 1M HCl. The Gly–His bond was then cleaved and the hippuric acid produced by the reaction was extracted with 1.5 ml ethyl acetate. Thereafter, the mixture was centrifuged to separate the ethyl acetate layer; then, 1 ml of the ethyl acetate layer was transferred to a clean test tube and evaporated. The residue was re-dissolved in distilled water and its absorbance was measured at 228 nm. The control experiment was performed without the test sample and the ACE inhibitory activity was expressed as percentage inhibition [17]:

$$\% \text{ Inhibition} = [(Abs_{Control} - Abs_{Samples})/Abs_{Control}] * 100$$

2.6 DPPH free radical scavenging ability

The free radical scavenging ability of all the extracts was evaluated as described by [18]. Briefly, appropriate dilutions of the extracts (1 ml) were mixed with 1 ml 0.4 mM methanol solution containing DPPH radicals; the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical scavenging ability of the extracts was subsequently calculated.

$$\text{DPPH free radical scavenging ability} = [(Abs_{ref} - Abs_{sample})/Abs_{ref}]*100$$

where Abs_{ref}= absorbance of the reference (reacting mixture without the test sample) and Abs_{sample}= absorbance of reacting mixture with the test sample.

2.7 Lipid peroxidation assay

The rats were decapitated under mild diethyl ether anaesthesia and the cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1/10, w/v) with about 10-up-and–down strokes at approximately 1200 rpm

in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000xg to yield a pellet that was discarded, and a low-speed supernatant (S1) which was kept for lipid peroxidation assay [19].

2.8 Lipid peroxidation and thiobarbibutric acid reactions

The lipid peroxidation assay was carried out using the modified method of [20]. 100 µl of S1 fraction was mixed with a reaction mixture containing 30 µl of 0.1 M (pH 7.4) Tris-HCl buffer, plantain peel extracts (0 to 100 µl) and 30 µl of the pro-oxidant (70 µM SNP or 250 µM $FeSO_4$), and the volume was made up to 300 µl by water before incubation at 37 °C for 1 h. The colour reaction was developed by adding 300 µl of 8.1% sodium dodecyl sulphate to the reaction mixture containing S1. This was subsequently followed by addition of 600 µl of acetic acid/HCl (pH 3.4) buffer and 600 µl of 0.8% thiobarbituric acid. This mixture was incubated at 100 °C for 1 h Thiobarbituric acid-reactive species (TBARS) produced were measured at 532 nm with JENWAY 6305 spectrophotometer, and the absorbance was compared with that of a standard curve using malondialdehyde (MDA).

2.9 Gas chromatography analysis

The qualitative-quantitative analysis of the phenolic compounds of the samples was carried out using the method reported by Kelley *et al.* [21]. The phenolic compounds were extracted from each sample as described by Kelley *et al.* [21] and Provan*et al.* [22]. After extraction, the purified extracts (1 µl; 10:1 split) were analysed for composition by comparison with phenolic standards (Aldrich Chemical Co., Milwaukee, W1) and a chromatography with standards on a Hewlett-Packard 6890 GC (Hewlett-Packard Corp., Palo Alto, CA) equipped with a derivatized non-packed injection liner, a Rtx-5MS (5% diphenyl-95% dimethyl polysiloxane) capillary column (30 m length, id 0.25 mm, 0.25 µm film thickness), and detected with a flame ionization detector (FID). The following conditions were employed for phenolic acid separation: injector temperature, 230 °C; detector temparature: 320 °C; temperature program, 80 ºC for 5 min, then ramped to 250 ºC at 30 ºC/min.

2.10 Data Analysis

The results of 3 replicate experiments were pooled and expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyse the means, and the post hoc treatment was performed using Duncan`s multiple test [23]. Significance was accepted at $P \leq 0.05$. EC_{50} (extract concentration causing 50% antioxidant activity) was determined using non-linear regression analysis.

3. RESULT AND DISCUSSION

3.1 ACE inhibition assay

The antihypertensive potentials of the extracts were assessed by characterizing the interaction of the aqueous extract of the peels with ACE which is considered to be a useful therapeutic approach in the management/treatment of high blood pressure. As shown in Fig. 1, the results revealed that all the extracts inhibited ACE activity in a dose-dependent manner (0-25 µg/ml). However, as revealed by the extract concentration causing 50% DPPH radical scavenging ability (IC_{50}) values, unripe plantain peel (UPP) extract (IC_{50} =14.93 µg/ml) had a stronger inhibition of ACE activity than over ripe plantain peel (OPP) extract (IC_{50} = 21.80 µg/ml) and ripe plantain peel (RPP) extract (IC_{50} = 21.32 µg/ml) as shown in Table 1. The UPP had a 75.46% ACE inhibition at the concentration of 25 µg/ml compared with the OPP extract which had an inhibition of 54.11% at the same concentration. ACE inhibitors have been widely developed to prevent angiotensin-II production in CVD patients, and have been utilized in clinical applications since the discovery of ACE inhibitors in snake venom [24]. The high inhibitory effects of the water-extractable phytochemicals could have contributed to the observed medicinal properties of the peels. This may, subsequently, be used for the development of nutraceuticals in hypertension management and/or prevention of hypertension.

3.2 1,1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging ability

Prevention of the chain initiation step by scavenging various reactive species, such as free radicals, is considered to be an important antioxidant mode of action. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) is a stable radical, and has been used to estimate the radical-scavenging capacities of antioxidants. This is based on a model system whereby antioxidant capacity is measured by the ability to donate a hydrogen atom, thereby neutralizing DPPH radicals [25, 26]. The extracts (unripe, ripe, and over ripe plantain peel) scavenged the DPPH free radical in a dose-dependent manner (0-333.33 µg/ml). The DPPH free radical scavenging ability of the extracts of the plantain peels is presented in Table 1 and Fig. 2; however, as revealed by the IC_{50} values in Table 2, UPP extract (IC_{50} = 218.43 µg/ml) had the highest DPPH scavenging ability while OPP extract (IC_{50} = 253.90 µg/ml) had the lowest one. A similar trend was observed in the DPPH radical scavenging ability of *Emblicaofficinalis* which had an IC_{50} value of 181 µg/ml [27].

3.3 Inhibition of Fe^{2+}- and SNP-induced lipid peroxidation

Lipid peroxidation in biological membranes is considered as one of the major mechanisms of cell injury in aerobic organisms subjected to oxidative stress [28]. The inhibition of Fe^{2+}-induced lipid peroxidation in isolated rat's heart homogenates by the extracts is presented in Fig. 3. Incubation of the rat's heart in the presence of Fe^{2+} caused a significant increase ($P<0.05$) in the MDA content of the heart (150.75%). These findings agree with our earlier reports on the interaction of Fe^{2+} with the brain [29], in which Fe^{2+} was shown to be a very potent initiator of lipid peroxidation (a pro-oxidant) in the brain. The increased lipid peroxidation in the presence of Fe^{2+} could be attributed to the fact that Fe^{2+} can catalyze one-electron transfer reac-

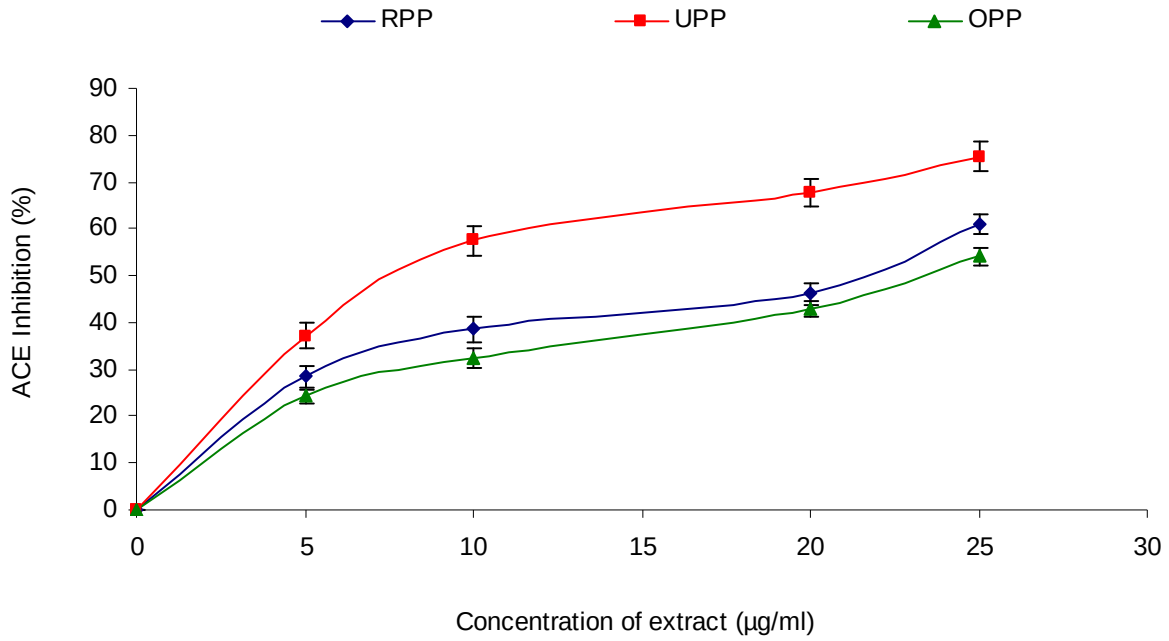

FIGURE 1 - Angiotensin I converting enzyme inhibitory activity of aqueous extract of plantain peels (*Musa paradisiaca*). Values are represented as means ± standard of triplicate experiments (UPP – Unripe plantain peel; RPP – Ripe plantain peel; OPP – Over-ripe plantain peel).

TABLE 1 - IC_{50} (concentration of sample causing 50 % enzyme inhibition) value of Angiotensin–I converting enzyme (ACE) inhibitory activity and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging ability by aqueous extract of plantain peels (µg/ml).

Amp	$_0$ of ACE Inhibitory activity (µg/ml)	IC_{50} of DPPH radical scavenging ability (µg/ml)
RPP	21.32 ± 3.2^a	230.41 ± 8.5^a
UPP	14.93 ± 1.2^b	218.43 ± 7.8^b
OPP	21.80 ± 2.5^a	253.90 ± 9.4^a

Values represent means ±standard deviation (n=3), Values with the same alphabet along the same column are not significantly different (P>0.05; UPP – Unripe plantain peel; RPP – Ripe plantain peel; OPP – Over-ripe plantain peel).

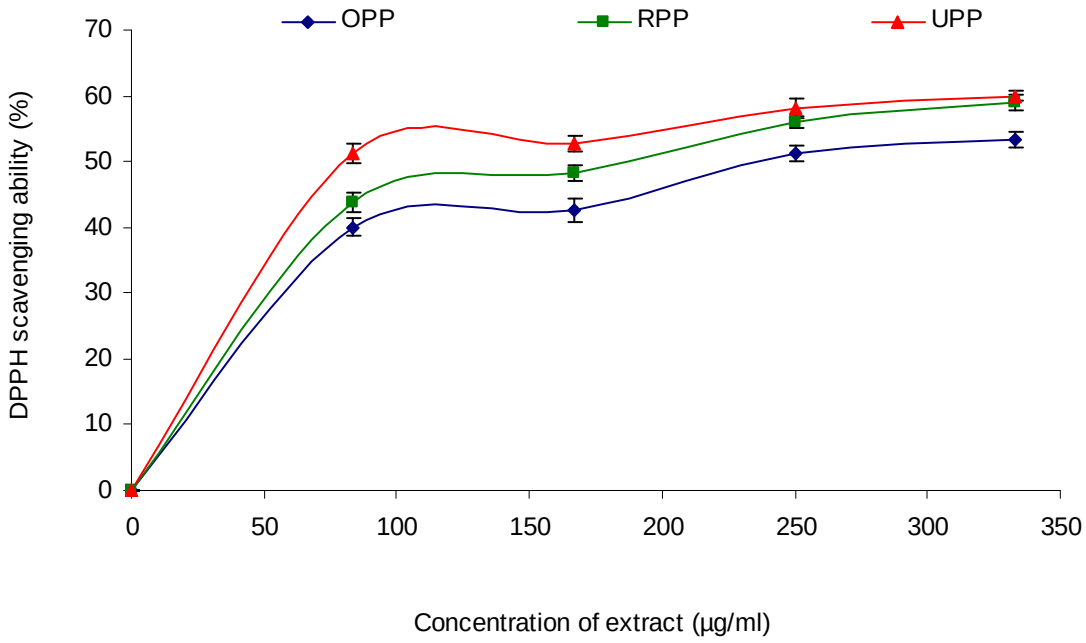

FIGURE 2 -DPPH radical scavenging ability of aqueous extract from unripe, ripe and over ripe plantain (*Musa paradisiaca*) peels. Values are represented as means ±standard of triplicate experiments (OPP – Over ripe plantain peel; RPP – Ripe plantain peel; UPP – Unripe plantain peel).

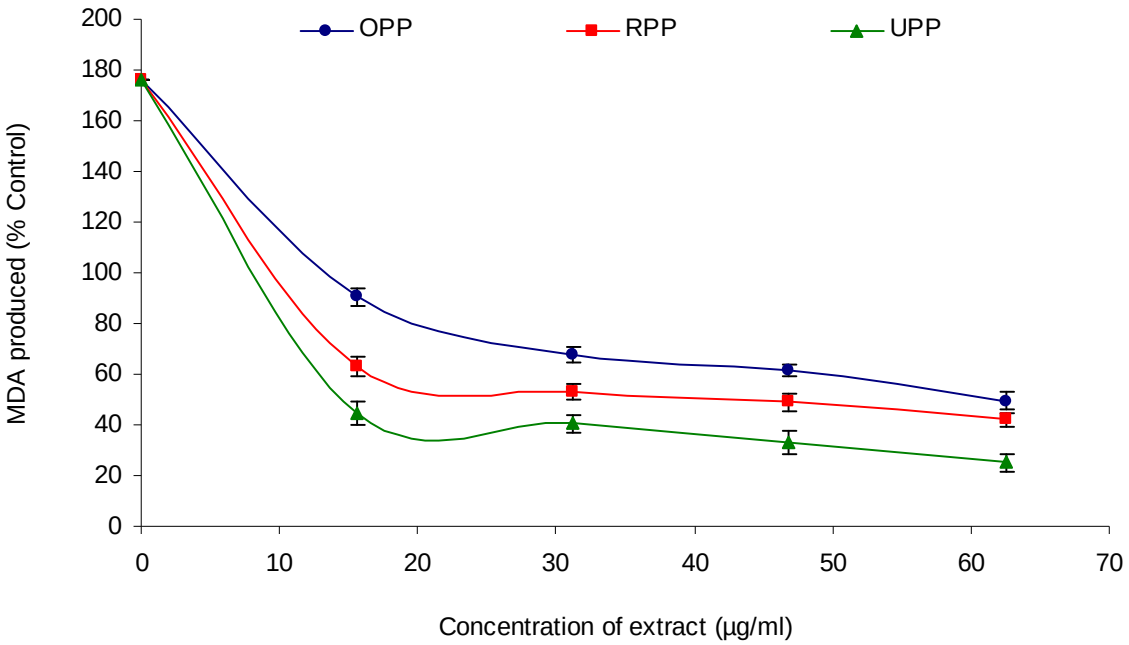

FIGURE 3 - Inhibition of Fe^{2+}-induced lipid peroxidation in isolated rat's heart homogenates by aqueous extract of plantain peels (*Musa paradisiaca*). Values are represented as means ±standard of triplicate experiments (OPP – Over ripe plantain peel; RPP – Ripe plantain peel; UPP – Unripe plantain peel).

TABLE 2 - EC_{50} value of inhibition of Fe^{2+} and sodium nitroprusside-induced lipid peroxidation in rats' hearts by phenolic extracts of plantain peels (µg/ml).

	EC_{50} of Fe^{2+}-induced lipid peroxidation (µg/mL)	EC_{50} of SNP-induced lipid peroxidation (µg/ml)
UPP	38.49± 2.6[a]	34.77 ± 2.7[a]
RPP	48.36 ± 4.7[b]	43.27 ± 3.4[b]
OPP	72 ± 5.3[c]	71.12 ± 5.7[c]

Values represent means ± standard deviation of triplicate readings. Values with the same letter along the column are not significantly (P<0.05) different; UPP – Unripe plantain peel; RPP – Ripe plantain peel; OPP – Over-ripe plantain peel).

tions that generate ROS, such as the reactive OH, which is formed from H_2O_2 through the Fenton reaction. Iron also decomposes lipid peroxides, thus generating peroxyl and alkoxyl radicals, which favor the propagation of lipid oxidation [30]. Malondialdehyde (MDA) is the end product of lipid peroxidation, a process where reactive oxygen species (ROS) degrade polyunsaturated fatty acids. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation end products. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism [31]. In this study, the introduction of extracts (0-62.5 µg/ml) from the plantain peels caused a significant dose-dependent decrease (P<0.05) in the MDA content of the Fe^{2+}-stressed heart homogenates; however, all the extracts inhibited MDA production in heart tissue in a dose-dependent manner (0–62.5 µg/ml) with the UPP extract (IC_{50} = 38.39 µg/ml) exhibiting the highest inhibitory effect while the OPP extract (IC_{50} = 72.00 µg/ml) had the least inhibitory effect (Table 2). The least MDA production (25.21 %) was occurring at the introduction of the highest concentration of the UPP extract (62.5 µg/ml) as shown in Fig. 3. The mode of inhibition of Fe^{2+}-induced lipid peroxidation cannot be categorically stated; however, there is the possibility that the water-extractable phytochemicals could have formed complexes with the Fe^{2+}, thereby preventing them from catalyzing the initiation of lipid peroxidation, or the phytochemicals could have scavenged the free radicals produced by the Fe^{2+}-catalyzed reaction [32].

Sodium nitroprusside (SNP) is a component of antihypertensive drugs commonly used in the management of hypertension. It causes cytotoxicity through the release of cyanide and nitric oxide (NO) [33]. The protective properties of the plantain peel extracts against SNP-induced lipid peroxidation in the heart could be because of the ability of the antioxidant phytochemicals present in the aqueous extract to scavenge the nitrous and Fe radicals produced from the decomposition of SNP. Likewise, incubation of rat's heart tissue homogenates in the presence of sodium nitroprusside (SNP) also caused a significant increase (P<0.05) in the rat

heart MDA content, as shown in Fig. 4; however, all the extracts inhibited MDA content in rat heart homogenate in a dose-dependent manner (0–62.5 µg/ml) as shown in Table 2. We found that aqueous extract of UPP had a higher inhibitory effect on MDA production in the heart *in vitro* (decreasing from 42.41 to 30.36%) than did OPP (a decrease from 89.53 to 57.59%). This result agrees with our findings, a high inhibitory effect on MDA [34, 35] . The protective properties of the UPP extract against sodium nitroprusside-induced lipid peroxidation in the heart could be because of the ability of the antioxidant phytochemicals present in the aqueous extract to quench-scavenge the nitrous radical and Fe produced from the decomposition of sodium nitroprusside.

3.4 Phenolic composition

It has been reported that high concentrations of polyphenols are present in the skin and seeds of fruits [36]. Polyphenols are secondary metabolites that plants produce to protect themselves from other organisms. Dietary polyphenols have been shown to play important roles in human health. Recent studies have revealed that many of these diseases are related to oxidative stress from reactive oxygen and nitrogen species. Polyphenols have been found to be strong antioxidants that can neutralize free radicals, chelate metallic ions, suppress the generation of free radicals, and act as direct radical scavengers of the lipid peroxidation chain reactions [32, 37, 38]. Nature has bestowed us with-many different kinds of plants, and all parts, individually

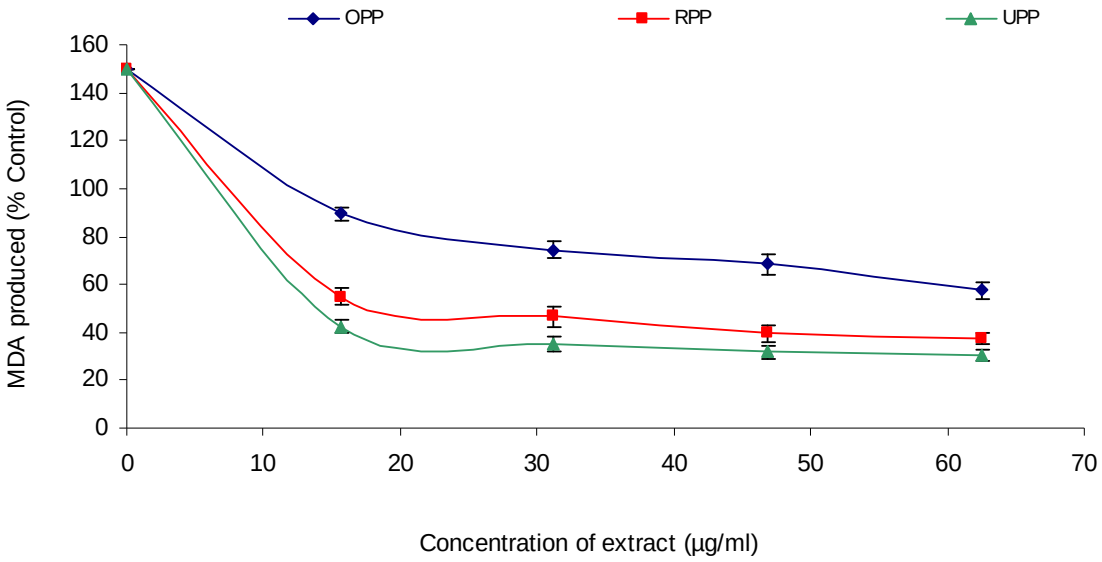

FIGURE 4 -Inhibition of SNP-induced lipid peroxidation in isolated rat's heart homogenates by aqueous extract of plantain peels (*Musa paradisiaca*). Values are represented as means ±standard of triplicate experiments (OPP – Over ripe plantain peel; RPP – Ripe plantain peel; UPP – Unripe plantain peel).

TABLE 3 -Phenolic composition of different plantain peels.

Phenolics	RPP (mg/100g)	OPP (mg/100g)	UPP (mg/100g)
Astragalin	173.80	211.7	186.90
Isoquercitin	90.54	91.51	114
p-Coumaric acid	75.88	70.75	64.42
Quercitin	73.07	89.55	109.90
Rutin	54.58	40.80	68.13
Catechin	22.17	50.61	24.96
Protocatechuic acid	12.04	23.64	12.82
Chlorogenic acid	11.47	12.61	19.41
Cavacrol	10.69	17.66	9.184
Caffeic Acid	96.02	ND	94.66
Eugenol	7.63	2.49	7.66
Vanillic Acid	2.33	7.16	2.07
Ferulic Acid	53.11	ND	67.42
Myricetin	7.81×10^{-1}	3.15×10^{-1}	1.13

UPP – Unripe plantain peel; RPP – Ripe plantain peel; OPP – Over-ripe plantain peel; ND – Not detected

or totally, exhibit therapeutic properties. The parts may be leaf, bark, seed, stem, flowers, fruits, twigs and peels etc. Each part shows different biological activity and antioxidant potency [39-42]. The plantain peel is not any different; it has also been bestowed by nature with some phenolic compounds that are profound antioxidants. Astragalin, isoquercetin, p-coumaric acid, quercetin, rutin, catechin, protocatechuic acid and carvacrol are the dominant phenolic compounds found in all the plantain peel (unripe, ripe, and over ripe) samples studied. These phenolic compounds have been reported to be responsible for the various biological effects including neuroprotective, cardioprotective, chemopreventive, antioxidant, anti-inflammatory, and anti-allergic properties [43].

4. CONCLUSION

The plantain peel extracts, especially the unripe plantain peel extract, had the highest scavenging ability, highest inhibition of angiotensin-I converting enzyme, and also protect the heart from Fe^{2+}- and SNP-induced lipid peroxidation *in vitro*. However the inhibitory activities and antioxidant properties can be attributed to the polyphenol content of the plantain peels. Moreover, the strong angiotensin-I converting enzyme inhibition makes it a good nutraceutical for the management of hypertension, with minimal side effects, currently observed with some of the drugs presently used for hypertension. Further work is ongoing in our laboratory to ascertain the effects of the plantain peels in animal models.

The authors have declared no conflict of interest.

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Received: August 24, 2014
Accepted: October 21, 2014

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ISOLATION AND ANTIBIOTIC RESISTANCE OF *Campylobacter* spp. FROM CHICKEN AND CATTLE MEAT IN TURKEY

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ABSTRACT

The aim of this study was to determine the level of antibiotic resistance by *Campylobacter* spp. isolates to 15 different antibiotics belonging to 8 classes, as investigated using the agar diffusion method. A total of 120 food samples were analysed (chicken and minced beef), and 71 of them (59.2%) were contaminated with *Campylobacter jejuni, Campylobacter coli,* or *Campylobacter lari*. Most of the isolates were resistant to ampicillin (95.7%), cefazolin (95.7%), and trimethoprim-sulphamethoxazole (90.1%). In contrast, most of the isolates showed a low incidence of resistance to meropenem (11.2%) and imipenem (5.6%). Multiple antibiotic resistance (MAR) index values ranged from 0.20-0.80. The results demonstrated that antibiotic-resistant *Campylobacter* spp. were easily recovered from chicken and cattle meat, and that multiple antibiotic-resistant strains may become a potential risk for public health.

KEYWORDS: *Campylobacter jejuni*, *Campylobacter coli*, food, antibiotic resistance, MAR index.

1. INTRODUCTION

In recent years, Gram-negative microaerophilic food-pathogenic *Campylobacter* species, found worldwide in different sources (water, various animals and humans), have become a major source of concern for food processing units and public health authorities in several countries. A common characteristic of *C. jejuni* is its ability to multiply at elevated temperatures and under microaerophilic (6% O_2, 7% CO_2, 7% H_2, 80% N_2) conditions [1]. Thermophilic *Campylobacter* species, particularly *Campylobacter jejuni* and *C. coli*, can easily colonise the gastro-intestinal system of most birds and mammals, and are the most often isolated *Campylobacter* species in humans with gastro-enteritis [2]. *Campylobacter* species are the primary cause of human intestinal disease of bacterial origin, as has been demonstrated in many developed countries [3]. More than 80% of these events are caused by *C. jejuni*, and approximately 10% by *C. coli*. Contamination with food-borne *Campylobacter* usually follows the ingestion of inappropriately handled or cooked food. *Campylobacter* remains one of the most common bacterial causes of diarrhoea in both industrialised and developing countries [4]. Major sources of *Campylobacte*r infection in humans are, in particular, poultry and poultry products. Ready-to-eat food products, undercooked poultry consumption and direct hand-to-mouth transfer are vectors behind the spread of *Campylobacter* spp. [5]. Campylobacteriosis is referred to as a zoonotic disease, with many animals, including poultry and pigs, acting as a *Campylobacter* source. Birds are the main sources of *C. jejuni* [6]. In animal farms, different antibiotics are added to feed to prevent infectious disease but also as growth factor additives. The wide use of antibiotics in poultry has led to the emergence of food-borne antibiotic-resistant pathogens [7]. Most cases of campylobacteriosis are self-limiting and do not require antimicrobial treatment, but in severe cases or instances of relapse medication is required, with the antibiotic choice for treatment being the macrolide erythromycin [8]. In patients for whom treatment is indicated, fluoroquinolones are often recommended [9]. In the case of systemic infections, an aminoglycoside, such as gentamicin or imipenem, is used [8].

Very little published work exists on the antibiotic resistance of *Campylobacter* isolated from the meat of chicken and cattle in Turkey, compared with hospital studies. *Campylobacter jejuni* and *Campylobacter coli* of chicken origin have previously been investigated and identified using polymerase chain reaction (PCR) in one study [10]. In another investigation, antibiotic susceptibility by *Campylobacter* spp. isolated from stools of children admitted to Hacettepe University Children Hospital was studied by Akan *et al.* [11]. Because of the low price of chicken meat (2,5 US $ per kg), it is an important public dietary protein source in Turkey and, consequently, foodborne chicken-associated pathogens cannot be ignored. Chicken farms are widespread in Turkey, and the chicken industry provides employment for those in chicken processing, and other forms of business directly or indirectly linked to chicken. Turkey is the largest producer of chicken meat in Europe. Its current annual chicken meat production is estimated to be approximately 1,723.905 tonnes [12].

Adana is the fifth largest city in Turkey; it is located in the south of Turkey and has a mild climate. In the summer,

the average air temperature is 27.4 °C. Favourable air conditions provide an advantage to several microorganisms that are responsible for food poisoning including *Campylobacter*, *Salmonella*, *Listeria* and *Staphylococcus* genera. Therefore, foodborne illness increases in the summer. The present study is the first work to determine the prevalence and resistance to antimicrobial agents of *Campylobacter* spp. isolated from Adana, Turkey.

The objectives of present study were to (i) identify the *Campylobacter* strains isolated from foods, (ii) evaluate the susceptibility patterns of these bacteria against antibiotics widely used in Turkey, and (iii) evaluate multiple-antibiotic resistance (MAR) index for all isolates. This study was carried out to obtain information on the epidemiology and resistance rates to antibiotics of *Campylobacter* spp. isolated from the meat of chicken and cows in southern Turkey.

2. MATERIALS AND METHODS

2.1 Collection of samples

A total of 120 food samples were collected from September 2013 to March 2014 from 60 different dairies, supermarkets, retail stores, and local butchers in Adana, Turkey. The numbers of samples were as follows: 35 chicken breasts, 35 chicken skins, 25 chicken wings, and 25 samples of minced beef.

All samples were transported to the laboratory in separate insulated cool boxes containing ice packs and were processed within 4 h of being collected.

2.2 Isolation of *Campylobacter* species

Isolation of *Campylobacter* spp. was carried out using enrichment and subsequent plating. Ten grams of each sample was added to 90 ml of Bolton selective enrichment broth (Oxoid, CM0983) supplemented with Bolton broth selective supplement (Oxoid, SR0183). The cultures were incubated at 42 °C for 48 h in an anaerobic incubator containing a gas mixture of 10% CO_2, 5% O_2, and 85% N_2. After incubation, 10 µl were streaked onto mCCDA plates (Merck, 100070) supplemented with CCDA selective supplement (Merck, 100071, and plates were incubated at 42 °C for 48 h under microaerobic conditions. From each mCCDA plate, one or two presumptive *Campylobacter* colonies were picked and purified on 7% blood agar (Columbia Blood Agar Base, Oxoid, CM0331 supplemented with defibrinated horse blood).

One hundred and thirty-one presumptive *Campylobacter* isolates were identified to the species level using the following phenotypic tests to distinguish between *C. jejuni*, *C. coli* and *C. lari*; Gram staining and determination of oxidase and catalase activity, nitrate reduction, hippurate hydrolysis, urease activity, H_2S production on triple sugar iron (TSI) slants, growth at 42 °C, sensitivity to nalidixic acid and cephalothin, growth at 25 °C, and growth in the presence of 1% glycine or 3.5% NaCl.

2.3 Antibiotic resistance testing

Antimicrobial susceptibility testing was performed via an agar diffusion test [13], using Mueller–Hinton agar (Oxoid, CM0337) and 15 antibiotic discs representing 8 classes of antibiotics. The antibiotics and their sensidisk concentrations were as follows: aminoglycosides: amikacin (AN, 30 µg), gentamicin (GM, 10 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg); carbapenems: meropenem (MEM, 10 µg), imipenem (IPM, 10 µg); cephalosporins: cefazolin (CZ, 30 µg), cefepime (FEP, 30 µg), ceftizoxime (ZOX, 30 µg), cefuroxime (CXM, 30 µg); nitrofurans: nitrofurantoin (F/M, 300 µg); chloramphenicols: chloramphenicol (C, 30 µg); penicillins: ampicillin (AM, 10 µg); tetracyclines: tetracycline (TE, 30 µg); and trimethoprim-sulphamethoxazoles: trimethoprim-sulphamethoxazole (SXT, 1.25 and 23.75 µg). The isolates were considered to be sensitive according to the manufacturer's instructions (BBL, MD, USA).

Reference strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, as recommended by NCCLS [14], were used as control organisms for verification of the antibacterial effect of the discs. All discs were purchased from Becton–Dickinson (BBL, MD, USA).

For all isolates, MAR index values were calculated as a/b, where 'a' represents the number of antibiotics the isolate was resistant to, and 'b' represents the total number of antibiotics the isolate was tested against. A MAR index value >0.2 is observed when isolates are exposed to high-risk sources of human or animal contamination, where antibiotics use is common. In contrast, a MAR index value < or = 0.2 is observed when antibiotics are seldomly or never used [15].

3. RESULTS AND DISCUSSION

3.1 Campylobacter spp. in foods

A total of 71 isolates of *Campylobacter* spp. were recovered from the four types of food (40 (56.3%) strains of *C. jejuni,* 21 (29.6%) of *C. coli* and 10 (14.1%) of *C. lari*). Rahimi *et al.* [16], in a study performed in Iran, reported that the most prevalent *Campylobacter* species isolated from meat samples was *Campylobacter jejuni* (88.3%), with the remaining isolates being *Campylobacter coli* (11.7%). In the present study, the percentage of positive samples varied markedly between different foods as follows: chicken breasts 65.7%, chicken skin 51.4%, chicken wings 64.0%, and minced beef 56.0% (Table 1). A high incidence of *Campylobacter* spp. in ducks in Tanzania was also found by Nonga and Muhairwa [17] (80.0%), Parisi *et al.* [18] (73.0% in raw chicken samples in Italy), and Ghimire *et al.* [19] (38.8% of 139 in dressed porcine carcass samples in Nepal).

Food processing units can become contaminated with *Campylobacter* spp. from equipment, contaminated raw materials, personnel and traffic. Although *Campylobacter* spp. has been isolated from many different types of raw and

processed food [20-23], molecular studies also suggested a link between the *Campylobacter* spp. found in farm environments and those causing diseases in local communities [24]. Many different animal species maintain *Campylobacter* spp. with no clinical signs [25]. Wild birds have been found with *Campylobacter* spp., with prevalence up to 50% in birds near chicken houses [26] and 40% in dead wild birds found in broiler houses [27]. There have been many studies on *Campylobacter* spp. in food animals and foods of animal origin in developed countries. In the past, research on cattle was limited, but has been increasing as outbreaks of human campylobacteriosis have been traced to foods of cattle origin [28].

3.2 Antibiotic resistance patterns

In the present study, among all of the isolates, a high percentage of bacteria were resistant to cefazolin (95.8%), ampicillin (95.8%), trimethoprim-sulphamethoxazole (90.1%), cefuroxime (81.7%), streptomycin (74.6%), and chloramphenicol (57.7%) (Table 2). The high degree of resistance to ampicillin in the present study was similar to the findings of Usha *et al.* [21] who observed a high incidence of ampicillin resistance (100%) in *Campylobacter jejuni* isolates from retail broiler chicken.

In the present work, the resistance trend against the different antibiotics for all isolates was as follows: AM = CZ>SXT>CXM>S>C>GM>K>AN>FEP>TE>ZOX>FM >MEM>IPM. The high degree of resistance to ampicillin in the present study was similar to the findings of Adzitey *et al.* [22]. They found a total of 76 ampicillin-resistant *Campylobacter jejuni* isolates among 94 isolates (81.0%) in their study.

Antibiotic resistance in *Campylobacter* species is due the acquisition of self- transferable and mobilisable plasmids. Tetracycline resistance was encoded by a 33 or a 41 Mda plasmid, and was transferred by conjugation [29].

Resistance to other antibiotics was relatively infrequent: ceftizoxime (22.5%), nitrofurantoin (21.1%), meropenem (11.3%), and imipenem (5.6%).

There were some marked differences in the degree of antibiotic resistance between samples. Isolates from chicken wings and minced beef showed higher resistance to cefazolin (100.0%) than did those from other samples. Concurrently, isolates from chicken skin and chicken wings showed higher resistance to ampicillin (100.0%) than did those from other samples. All isolates from minced beef were susceptible to imipenem. In contrast, isolates from other samples showed variable resistance to antibiotics (Fig. 1).

The use and misuse of antibiotics contribute to the development of resistance among bacteria including food-borne pathogens [30]. In addition, uncontrolled antibiotic use in animals used for food has led to the emergence of antibiotic resistance in food-borne pathogens including *Campylobacter,* and the transfer of these resistant organisms to humans through the food-chain.

TABLE 1 - Incidence of *Campylobacter* species in different foods in Turkey.

Type of food	No of samples	No and (%) of positive samples	No of C. *jejuni* strains	No of *C. coli* strains	No of *C. lari* strains
Chicken breasts	35	23 (65.7)	11	7	5
Chicken skin	35	18 (51.4)	10	6	2
Chicken wings	25	16 (64.0)	11	3	2
Minced beef	25	14 (56.0)	8	5	1
Total	120	71 (59.2)	40	21	10

TABLE 2 - Percentage of *Camylobacter* species resistant to 15 antibiotics belonging to 8 classes.

Classes of antibiotics	Antibiotics	*C. jejuni* N=40	*C. coli* N= 21	*C. lari* N=10	Average Resistances N=71
Aminoglycosides	Amikacin (AN, 30 µg)	15 (37.5%)	7 (33.3%)	3 (30.0%)	25 (35.2%)
	Gentamicin (GM, 10 µg)	26 (65.0%)	10 (47.6%)	4 (40.0%)	40 (56.3%)
	Kanamycin (K, 30 µg)	14 (35.0%)	8 (38.1%)	6 (60.0%)	28 (39.4%)
	Streptomycin (S, 10 µg)	30 (75.0%)	14 (66.7%)	9 (90.0%)	53 (74.6%)
Carbapenems	Meropenem (MEM, 10 µg)	5 (12.5%)	2 (9.5%)	1 (10.0%)	8 (11.3%)
	Imipenem (IPM, 10 µg)	3 (7.5%)	1 (4.8%)	-	4 (5.6%)
Cephalosporins	Cefazolin (CZ, 30 µg)	40 (100.0%)	19 (90.4%)	9 (90.0%)	68 (95.8%)
	Cefepime (FEP, 30 µg)	10 (25.0%)	7 (33.3%)	2 (20.0%)	19 (26.8%)
	Ceftizoxime (ZOX, 30 µg)	12 (30.0%)	3 (14.3%)	1 (10.0%)	16 (22.5%)
	Cefuroxime (CXM, 30 µg)	35 (87.5%)	17 (80.9%)	6 (60.0%)	58 (81.7%)
Nitrofurans	Nitrofurantoin (F/M, 30 µg)	8 (20.0%)	5 (23.8%)	2 (20.0%)	15 (21.1%)
Chloramphenicols	Chloramphenicol (C, 30 µg)	23 (57.5%)	13 (61.9%)	5 (50.0%)	41 (57.7%)
Penicillins	Ampicillin (AM, 10 µg)	38 (95.0%)	21 (100.0%)	9 (90.0%)	68 (95.8%)
Tetracyclines	Tetracycline (TE, 30 µg)	6 (15.0%)	8 (38.1%)	3 (30.0%)	17 (23.9%)
Trimethoprim-sulphamethoxazoles	Trimethoprim-sulphamethoxazole (SXT, 1.25 and 23.75 µg)	37 (92.5%)	19 (90.5%)	8 (80.0%)	64 (90.1%)

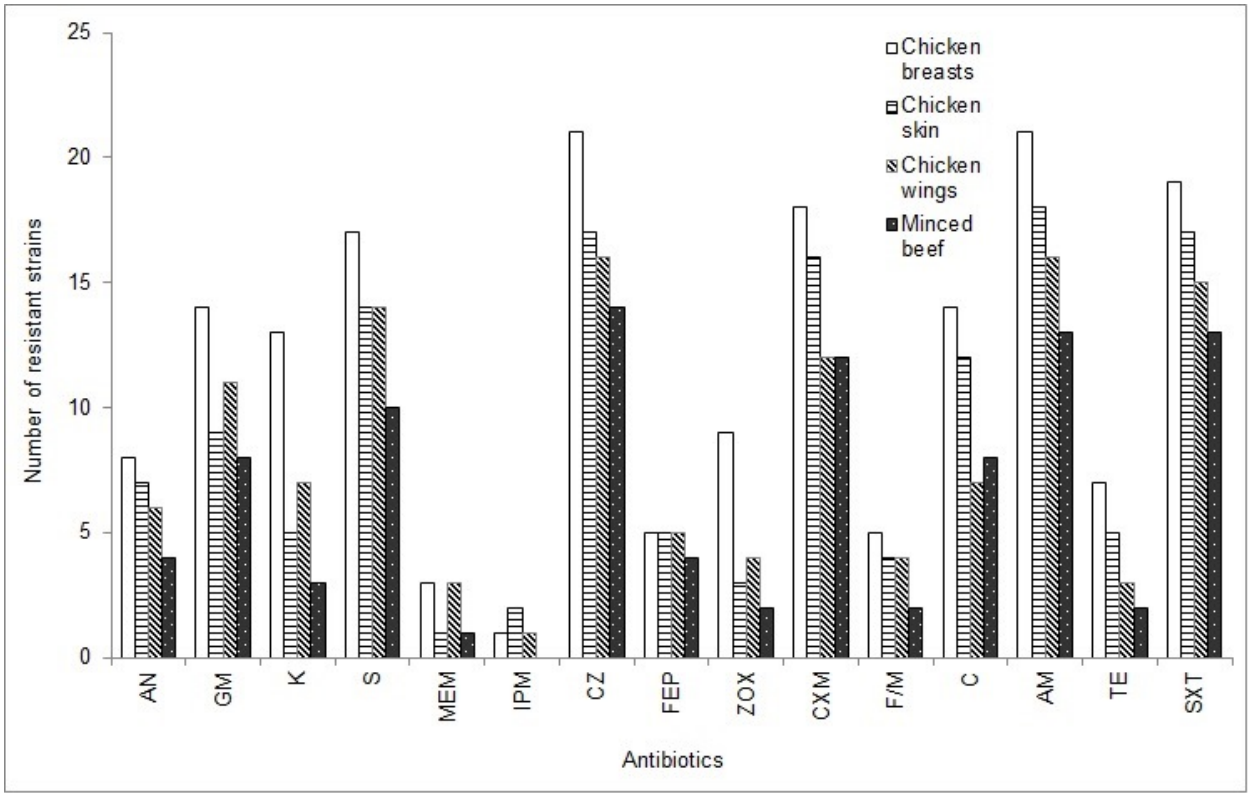

FIGURE 1 - Antibacterial resistance of *Campylobacter* spp. isolated from four different foods in Adana, Turkey.

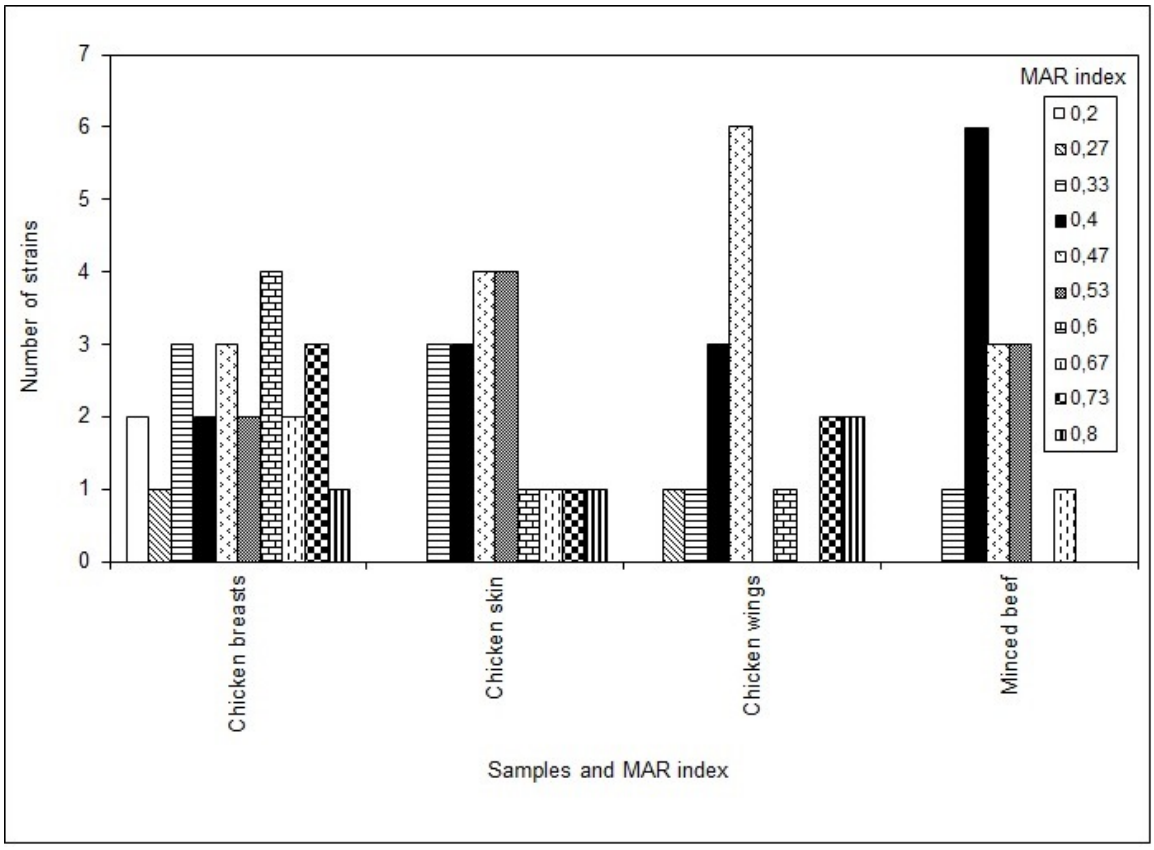

FIGURE 2 - Multi-antibacterial resistance of *Campylobacter* spp. isolated from four different foods in Adana, Turkey.

3.3 Multiple antibiotic resistance index

Bacterial isolates resistant to three or more antibiotics were designated as multiple-antibiotic-resistant. The multiple antibiotic resistance (MAR) index values ranged from 0.20 to 0.80 for the isolates. Resistance to 7 antibiotics was most common among the isolates, 16 isolates (22.5%) were resistant to 7 antibiotics (6, 4, 3, 3, from chicken wings, chicken skin, chicken breasts and minced beef sample, respectively); furthermore, 14 (19.7%) isolates were resistant to 6 antibiotics, and 9 (12.7%) to 8 antibiotics (data not shown).

The MAR index values ranged from 0.2 to 0.8 for chicken breast isolates, from 0.33 to 0.8 for chicken skin isolates, from 0.27 to 0.8 for chicken wing isolates, and from 0.33 to 0.67 for minced beef isolates (Fig. 2). Sánchez *et al.* [31] found a 95% resistance to one or more antimicrobial agents for *Campylobacter* isolates in the United States.

Many studies have associated multidrug resistance in *Campylobacter* spp. with the expression of the *cmeABC* efflux system [32, 33]. In *Campylobacter* spp., the efflux pump extrudes a broad range of structurally unrelated antibiotic agents (including fluoroquinolones, β-lactams, tetracycline, gentamicin) out of bacterial cells, and thus contributes to the intrinsic antibiotic resistance and high levels of multiple resistance [34]). There has been an increase in antibiotic resistance among *Campylobacter* spp. This is in line with a general worldwide pattern of increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria [35]. High percentages of resistant strains of *Campylobacter* spp. have been reported in dairy and meat products [18, 36], and because the incidence of contamination of these bacteria is high in chicken and beef products in Adana, Turkey, these foods are of particular concern.

In Finland, Schönberg-Norio *et al.* [37] examined the risk factors for *Campylobacter* infections. They found a total of 100 patients involved in the survey to be infected with *C. jejuni* or *C. coli*. All cases were sporadic and not associated with known outbreaks. They identified 3 major risk factors: (1) tasting or eating undercooked or raw meat, (2) drinking water from a dug well, and (3) swimming in water from natural sources. Private untreated water supplies were found to present a significant risk factor.

The first *Campylobacter* may have been isolated in 1913 (classified as *Vibrio* spp. until the genus *Campylobacter* was established in 1963) [38]. Increasing antimicrobial resistance in both medicine and agriculture in *Campylobacter* is recognised by various national authorities including the World Health Organization (WHO) as a major emerging public health concern [39].

There were numerous multiple antibiotic isolates, a situation of major public health concern because of Campylobacteriosis. The increasing demand for minimally processed foods along with consumer awareness of potential health risks associated with traditional preservatives has fuelled research examining new strategies for food preservation [40]. For example, some endemic herbs that have antibacterial activity are used as food ingredients in Iran [41].

Therefore, new intervention strategies are needed for meat and poultry products to better protect consumers from this pathogen. *Campylobacter* may be avoided by vigorous cleaning and sanitation, and contamination prevention programs; food safety and HACCP programs must be required in all plants. Strict standards for food safety practice are urgently needed. In recent years, modified atmosphere or vacuum packaging has been a common packaging technique used by the meat and poultry industry to extend the shelf-life of meat or meat products. Furthermore, irradiation has been well established as an antibacterial treatment to reduce pathogens on poultry products. It is also important to reduce or eliminate the incorrect therapeutic use of antimicrobial agents in veterinary science and human medicine. Furthermore, studies are needed to elucidate the mechanisms of antibiotic resistance among *Campylobacter* isolates.

ACKNOWLEDGEMENT

Thanks are extended to Cukurova University, Scientific Research Projects Fund (Project number: EF2012BAP12) for its financial support.

The authors have declared no conflict of interest.

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Received: September 24, 2014
Accepted: November 18, 2014

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SILICA NANOPARTICLES CAN INDUCE APOPTOSIS VIA DEAD RECEPTOR AND CASPASE 8 PATHWAY ON A549 CELLS

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ABSTRACT

Synthetic amorphous silica (SAS) is an important material that is widely used in different applications, such as medicine and industry. Although SAS is generally regarded as potential toxic, the cytotoxicity, pathogenicity and carcinogenicity of SAS nanoparticles have been recently investigated. In this study, we aim to investigate the ability of induction of cytotoxicity and apoptosis by SAS NPs on human NSCLC cell line A549 with potential mechanisms. SAS NPs (6, 15, 30 nm size; concentration ranges 0.1, 10, 250, 500 and 1000g/ml) have ability to induce cytotoxicity on A549 cell line. Among 6, 15, 30 nm size of SAS NPs, only 6 nm SAS NP has the ability to induce strong cellular cytotoxicity and induction of apoptosis on A549 cells. qRT-PCR results showed that 6 nm-sized SAS NP treatment also induces the expression levels of FADD, TNFR, BID and Caspase 8, and mRNAs. In conclusion, this study demonstrates that among 6, 15 and 30 nm size of SAS NPs, only 6 nm-sized SAS NP has strong cytotoxic effects on A549 cells, and this cytotoxicity comes from dead receptor-mediated induction of apoptosis on A549 cells.

KEYWORDS:
NSCLC, Synthetic amorphous silica (SAS), Apoptosis, terminal transferase dUTP nick end-labeling (TUNEL), Caspase

1. INTRODUCTION

Nanotechnology originates from the Greek word meaning "dwarf". A nanometer is one billionth (10^{-9}) of a m, which is tiny, and only the length of 10 hydrogen atoms. Nanoscience and nanotechnology are the study and application of extremely small things, and can be used across all the other scientific fields, such as chemistry, biology, physics, materials science, and engineering.

Nanotechnology industry is rapidly growing because of the physical and chemical properties of nanomaterials. The size of nanoparticles (NPs) is between 1-100 nm. The ultra-small size and unique properties of nanomaterials have led to increasing concerns about their potential toxicity. The NPs can easily pass some biological barriers according to their dimensions. Little is known about the cytotoxicity mechanisms of NPs.

Nanoparticles (NPs) are used in different applications including targeted drug delivery systems [1, 2], rapid diagnostics [3, 4] and biomolecular sensing [5], antimicrobial agents [6], stain-resistant clothing [7], and fluorescent labels [8], such as nanoparticle-based cancer therapeutics [9].

Even though studies have demonstrated that cancer cells and epithelial cells are capable of taking up nanoparticles [10, 11], the mechanism for the biological responses to NPs are still not well understood. To understand the transmigration of NPs into cells could enable the control over cellular uptake, and to predict the possible toxic effects. Some reports have shown that NPs are taken up by cells via non-endocytic pathways [12], and model membranes have indicated possible mechanisms for non-endocytic uptake [13].

Nano-materials have important roles on apoptosis by inducing apoptotic pathways [14]. Apoptosis is termed as a form of cellular suicide, and it has two pathways (extrinsic and intrinsic ones). Extrinsic pathway-mediated induction of apoptosis is caspase-dependent and stimulated by extracellular stress signals, which are sensed and propagated by specific transmembrane receptors. Intrinsic pathway-mediated induction of apoptosis needs some intracellular stress conditions like DNA damage, oxidative stress, etc. [15]. The size of NPs is very important on cellular damages, and the small-sized NPs are more effective to enter the cells than the large-sized ones [16].

SAS NPs are suitable materials for biomedical applications. They have been used in cancer therapy, DNA/drug delivery and enzyme immobilization research studies [17-20]. SAS NPs can induce reactive oxygen species genera-

tion, DNA damage and aberrant nucleoplasmic protein aggregation [21, 22]. Silica NPs are not only particle size-dependent [23, 24], but also affect surface charge [25].

Several studies have shown that SAS NPs are distributed in blood, spleen, kidneys, lungs, pancreas, or other organs [26, 27]. *In vitro* studies reported that SAS nanoparticles have cytotoxic effects on human embryonic kidney cells, endothelial cells, HepG2 cells and human bronchoalveolar carcinoma-derived cells [23, 28-30].

In this study, we aim to investigate the ability of induction of cytotoxicity and apoptosis by SAS NPs on human NSCLC cell line A549 with potential mechanisms.

2. MATERIALS AND METHODS

2.1 Cell culture

A549 were grown at 37 °C, in 5% CO_2 atmosphere, and were routinely maintained in Dulbecco's Modified Eagle Medium (DMEM; ingredients: 1 g/L D-glucose, 2 mM L-glutamin, 1 mM sodium pyruvate) supplemented with 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.2 Characterization of NPs

SAS NPs were purchased from H.C. Starck GmbH & Co (Germany, Leverkusen). Synthetic amorphous silica (6 nm) was previously used by Demir *et al.* [32]. Characterization of SAS NP is detected via typical transmission electron microscopy (TEM) image, DLS and LDV. The overview of properties of different nano-sized SiO_2 NPs are given in Table 1.

TABLE 1 - Overview of properties of different nano-sized SAS nanoparticles.

Particle size	6	9	15	30	55
Concentration (%)	15	30	30	45	50
Density (g/cm^3)	1,1	1,208	1,205	1,343	1,390
Ionicity	anionic	anionic	anionic	anionic	anionic
pH value	9	10	9	10	9

2.3 Cell viability assay

When the A549 cells reach 90% confluence, the medium was removed, then cells were washed with phosphate buffered saline, trypsinized, counted with a hemocytometer, and seeded into 96-well plates (3 x 10^4 cells/ml). After a 24-h incubation at 37 °C in 5% CO_2, the medium was removed and cells were treated with differently sized (6, 15, 30 and 55 nm) SAS nanoparticles in different SAS NPs concentrations (0.1, 10, 100, 250, 500, 1000 µg/ml) for 72 h. At the end of the incubation period, cell death was determined by a limunometric method using a CytotoxGlo kit (Promega, Madison, USA).

2.4 Terminal transferase dUTP nick end-labeling apoptosis analysis

A549 cells were trypsinized, counted with a hemocytometer, and then seeded into flasks (3 x 10^4/ml). For the detection of the SAS NPs-mediated induction of apoptosis, A549 cells were treated with IC_{50} values of 6-nm SAS (119.82 µg/ml) for 24 h at 37 °C in a humidified incubator with 5% CO_2. At the end of the incubation period, late apoptotic events were analyzed by terminal transferase dUTP nick end-labeling (TUNEL) analysis using the In Situ Cell Death Detection Kit (Millipore, Billerica, MA, USA).

2.5 Real-time reverse transcriptase–polymerase chain reaction analysis

Quantitative real-time reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis was applied to determine relative mRNA levels in 6nm SAS NP-treated and untreated control groups of A549 cells. Cells were treated with IC_{50} values of 6nm SAS NP for 24 h at 37 °C in a humidified incubator with 5% CO_2. At the end of the incubation time, treated nanoparticle (119.82 µg/ml) and untreated control groups of cells were washed with ice-cold phosphate-buffered saline, and then total RNA from the A549 cell line was isolated as described previously [31]. Expression of apoptosis-related genes were assessed by quantitative real-time qRT-PCR analysis using a Fast Star Taqman Probe Master kit (Roche, Germany) and a Roche Light Cycler 480 (Roche, Germany) according to the manufacturer's protocol. Sets of TaqManprobe and primers were purchased from Roche (Table 2). A pre-developed TaqMan assay endogenous control of a ß-actin kit (Roche) was used for control. The relative mRNA levels were calculated using the comparative Ct method. Levels of Bcl2, bax, p53, Fadd, Tnfr1, survivin, caspase 8, caspase 9, and caspase 3 transcripts were evaluated. The transcript level of the ß-actin gene was used as the endogenous reference.

2.6 Statistical analysis

All experiments were performed in 3 replicates, and repeated independently to confirm the results. Significance of the differences in the means was determined using Student's t test and considering $P \leq 0.05$ to be statistically significant.

3. RESULTS

3.1. SAS (6nm) NPs characterizations

We characterized the SAS (6 nm) NPs as previously described [32]. Briefly, 6 nm SAS NP was characterized by using TEM to determine the size and the morphology of SAS (Fig. 1a). The majority of NPs were in spherical shape and no important agglomerations were observed following the dispersion protocol. The average hydrodynamic diameter and zeta potential of the SAS NP suspension was determined by DLS and LVD, respectively (Figs. 1b and 1c).

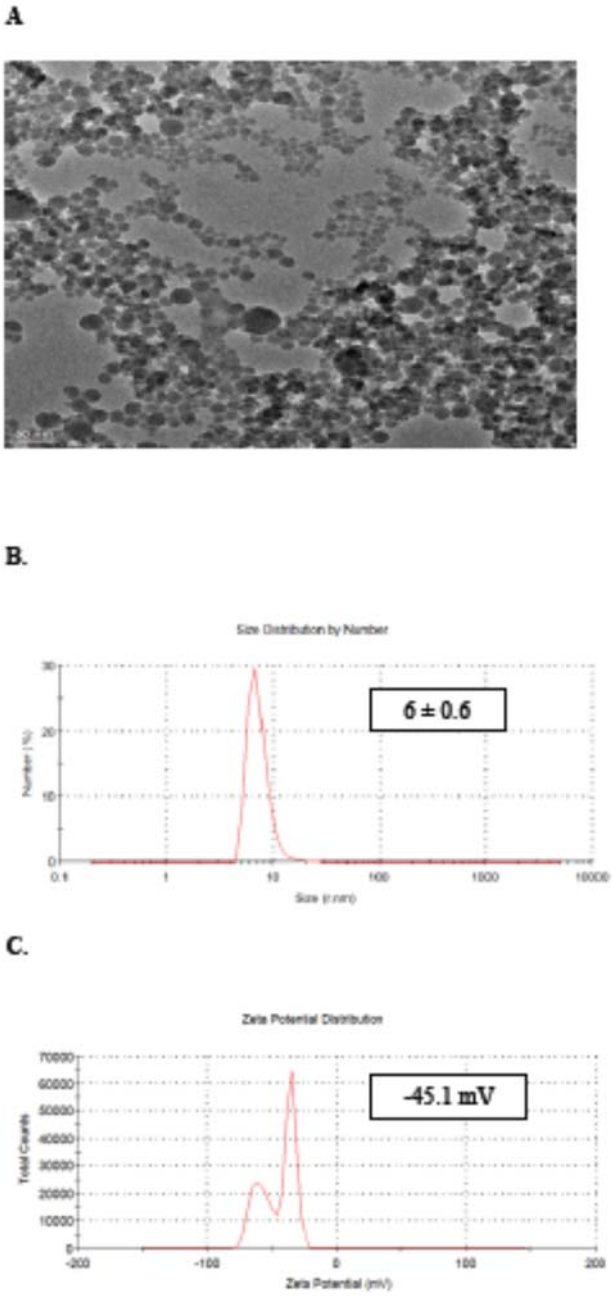

FIGURE 1 - Characterization of SAS NP (size 6 nm) (A), typical TEM image and size distribution histogram using such images (B), and (C) represents size distribution and zeta potential, by DCS and LDV characterization.

3.2. Anti-tumor effects of silica nanoparticles on A549 cells

We firstly examined different sizes (6, 15, 30 and 55 nm) and different concentrations (0.1, 10, 100, 250, 500 and 1000 µg/ml) of SAS NPs on A549 cells viability by using luminometric assay. As described in the method section, cells were treated with different sizes of SiO_2 for 72 h, and then cellular viability was measured. As seen in Fig. 2, among 6, 15, 30, and 55 nm sizes of SAS NPs, only 6-nm size had strong anti-tumor activity on A549 cells. It means that 6 nm-sized SAS NPs can induce dose-dependent cellular cytotoxicity on A549 cells.

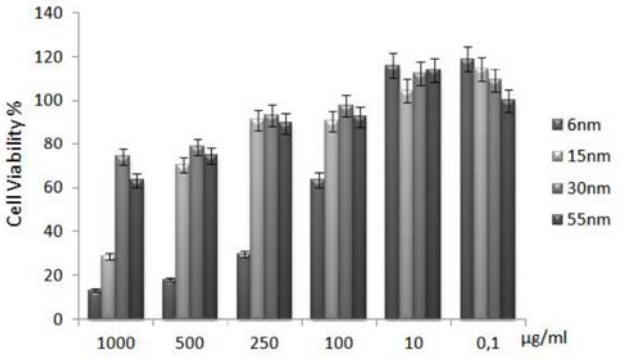

FIGURE 2 - Cytotoxic effects of 6, 15, 30, 55 nm sizes SAS NPs on NSCLC cells A549 (cells were seeded at a density of $3x10^4$/ml in 96-well plates. After 24 h, the cells were washed with phosphate-buffered saline, fresh growth medium was added, and then cells were treated with different sizes (6, 15, 30 and 55 nm) and different concentrations (0.1, 10, 100, 250, 500 and 1000 µg/ml) of SAS nanoparticles for 72 h. At the end of the incubation period, cell death was determined by the limunometric method.

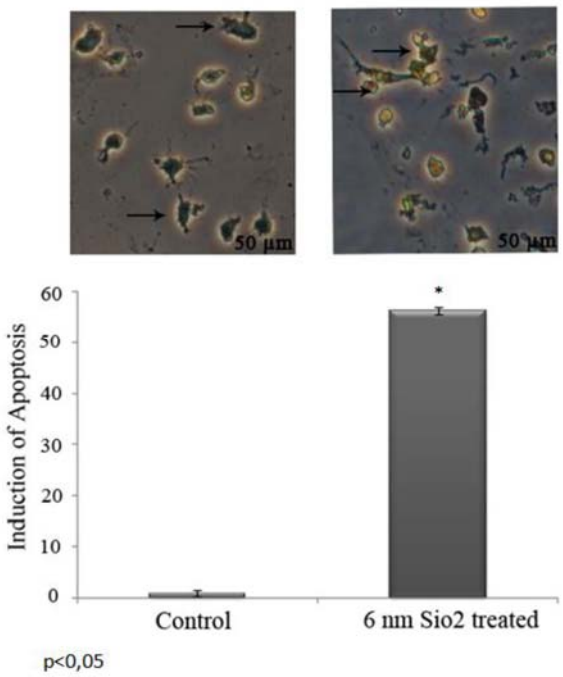

FIGURE 3 - SAS NPs (6 nm) strongly induces apoptosis on A549 cells (cells ($3·10^4$/ml) were incubated with IC_{50} (119.82 µg/ml) values of 6 nm-sized SAS NPs for 24 h. After the end of the incubation time, cells were washed with phosphate-buffered saline and then assayed by terminal transferase dUTP nick end-labeling analysis using an In Situ Cell Death Detection Kit (Millipore) system to indicate cellular apoptosis: (i) untreated control, (ii) cells treated with 6 nm-sized SAS NPs. Arrows indicate dark-stained nuclei, which indicate DNA fragmentation and nuclear condensation.

3.3. Induction of apoptosis on A549 cells by 6 nm SAS NP

Because we detected strong anti-proliferative effects of 6 nm SAS NPs on A549 cells, we wanted to examine its ability to induce apoptosis or not. A549 cells were treated with IC_{50} concentration of 6 nm SAS NPs (119.82 µg/ml) for 24 h, and then apoptosis was measured by TUNEL method. The TUNEL assay was developed as a method to

identify individual cells that were undergoing apoptosis by labeling the ends of degraded DNA with the polymerase terminal deoxynucleotidyl transferase. Fig. 3 clearly shows that 6 nm SAS NP treatment induced 56% cellular apoptosis on A549 cells when compared to untreated control group. For induction of apoptosis, there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. We examined which mechanisms are involved in 6 nm SAS NPs-mediated induction of apoptosis on A549 cells by qRT-PCR. We firstly treated A549 cells with IC_{50} concentration of 6 nm SAS NP (119.82 µg/ml) for 24 h, and then removed medium and isolated total RNA as described in method section. We wanted to examine if this induction of apoptosis is p53 or dead receptor-mediated. Therefore, we selected p53, survivin, FADD, TNFR, Bid, Caspase 3, 8, and 9 as a target; 6 nm SAS NP treatment led to induce cellular expression of FADD, TNFR, Caspase 8 and Bid, but not changed the expression of p53, Caspase 3, and 9 mRNA levels (Figs. 4, 5).

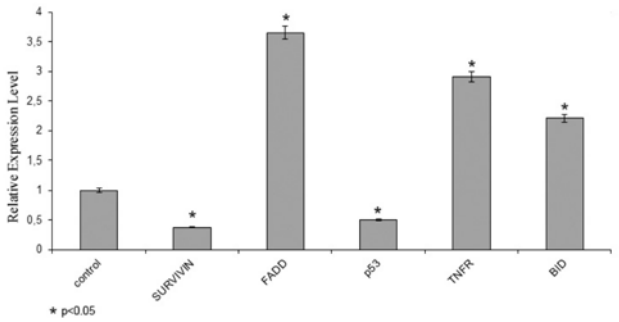

FIGURE 4 - The effects of SAS nanoparticle size (6 nm) on A549 cell surviving, p53, TNFR, FADD and BID mRNA expressions (cells were treated with IC_{50} concentration (119.82 µg/ml) of 6 nm SAS NP for 24 h. At the end of the incubation time, cells were washed with ice-cold PBS, total RNA isolated by trizol, cDNA synthesized by using ABM cDNA synthesis kit; then, surviving, p53, TNFR, FADD and Bid mRNA expression was evaluated by using specific probes. Data are mean – SD values of 3 analyses by quantitative reverse transcriptase–polymerase chain reaction).

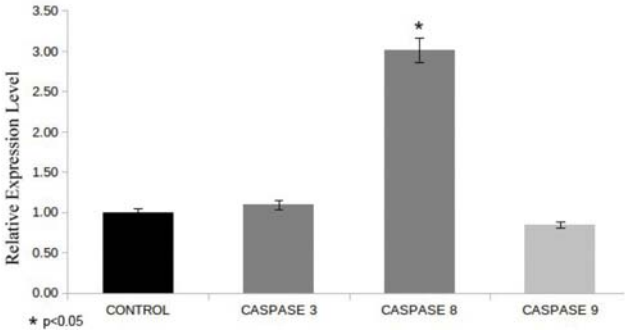

FIGURE 5 - The effect of SAS nanoparticle size 6 nm on A549 cell Caspase 3, Caspase 8 and Caspase 9 mRNA expressions (cells were treated with IC_{50} concentration (119.82 µg/ml) of 6 nm SAS NP for 24 h. At the end of the incubation time, cells were washed with ice-cold PBS, total RNA isolated by trizol method, cDNA synthesized by ABM cDNA synthesis kit; then, Caspase 3, Caspase 8 and Caspase 9 mRNA expression was evaluated by using specific probes. Data are mean – SD values of three analyses by quantitative reverse transcriptase–polymerase chain reaction).

This means that 6 nm SAS NPs-mediated induction of apoptosis is not through *p53*, but dead receptor pathway.

4. DISCUSSION

NPs are widely used in many applications, such as healthcare, gene theraphy, tissue engineering, and many industrial applications. Physicochemical characterization of nanoparticles is an important aspect of nano-toxicity research.

Up to date, there are few studies investigating the cytotoxic effects of nanoparticles, and no guidelines are presently available to quantify these effects, and there is some confusion in the literature. Many studies have shown that NP toxicity pathways depend on NP properties (e.g. size, charge and functionalization) [33]. Previous studies have described that different-sized SAS NPs effect different degrees of cytotoxicity with different mechanisms in the same A549 cell lines [34, 35]. Nowak *et al.* [35] reported that 20-nm SiO_2 NP has cytotoxic effects on A549 cells originating from induction of autophagy, but Ahamed [34] showed that the same-sized SiO_2 NP has also cytotoxic effect on A549 cells originating from induction of apoptosis. Because of this conflicting results, we aimed to investigate the cytotoxicity of SAS NPs (6, 15, 30 55 nm), with potential mechanisms of induction of apoptosis on A549 cell lines.

Our results clearly indicate that SiO_2 NPs has cytotoxic effects on A549 cell line, and this cytotoxicity depends on the size of SiO_2 NPs. Our results also indicate that 6 nm size of SiO_2 NPs has stronger cytotoxicity than 15, 30, or 55 nm size of SiO_2 NPs on A549 cell line; our cytotoxicity results are in good agreement with previously published results that small-sized SiO_2 NPs have stronger cytotoxicity when compared to bigger sizes of SiO_2 NPs [23, 27, 36, 37]. After observing the cytotoxicity effects of SiO_2 NPs, we wanted to examine the potential mechanisms of this cytotoxicity, with focus on the induction of apoptosis because several researchers have reported that SiO_2 NP can induce apoptosis in several human cancer cell lines, hepatic cell lines [27], dermal fibroblasts [36], and lung cancer cell lines [34]. Moreover, there are two different explanations for SiO_2 NPs-induced cytotoxicity in the same A549 cell lines, either from induction of apoptosis [36] or induction of autophagy [35]. Cell death may occur by two distinct mechanisms; apoptosis or necrosis. Apoptosis is an active physiological process resulting in cellular self-destruction that involves specific morphological and biochemical changes in the nucleus and cytoplasm [38, 39]. NPs suppress the proliferation of cancer cells by inducing apoptosis or other cell death mechanisms. To determine if SAS NPs-mediated cellular cytotoxicity comes from apoptosis or not, A549 cells were treated with SAS NP (6 nm) at the IC_{50} concentration (119.82 µg/ml), and TUNEL assay was used for determination of apoptosis on A549 cell line. Figure 3 clearly indicates that 6 nm-sized SiO_2 NPs can induce

apoptosis on A549 cells within 24 h. Then, we wanted to determine that 6 nm-sized SAS NPs induced apoptosis on A549 cells in p53 medium or not, by qRT PCR. We also wanted to examine the expression of surviving, FADD, TNFR, and BID to evaluate potential mechanisms of SAS NP-mediated induction of cellular cytotoxicity. Figure 4 shows that 6 nm-sized SAS NP treatment led to decrease of p53 surviving expression but induced the expressions of FADD, TNFR and BID; these result give us clues that 6 nm-sized SAS NP cytotoxicity comes not from p53 but dead receptors on A549 cells.

It is well-known that there are two mechanisms for the induction of apoptosis, the receptor-mediated extrinsic through caspase 8, and the mitochondrial intrinsic through caspase 9 [40]. Our qRT PCR results have shown that 6 nm-sized SAS NPs treatment increased caspase 8 mRNA expression but not that of caspase 3 and caspase 9 (Fig. 5). Thus, 6 nm-sized SAS NP cytotoxicity comes from dead receptor-mediated induction of caspase 8. Our results are not in good agreement with Ahamed [34] or Nowark *et al.* [35], because these authors reported that SiO_2 NP induces expression of caspase 3 and caspase 9.

Our results are supporting our hypothesis because caspase 9 is only partly involved in mitochondrial pathway for induction of apoptosis. After 6-nm SAS NP treatment, no change of caspase 9 level was observed; mRNA expression surviving and Bid expression were not increased but decreased, which means that 6 nm-sized SAS NP induces apoptosis not on mitochondrial pathway.

5. CONCLUSION

In conclusion, we have shown that SAS NPs produce significant cytotoxicity to A549 cells in dose- and size-dependent manner, especially 6 nm SAS with an IC_{50} value of 119.82 μg/ml. Furthermore, SAS (6 nm) NP initiate apoptosis through extrinsic pathway. Finally, these NPs still need to be investigated in future studies for the use of cancer therapy.

The authors have declared no conflict of interest.

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Received: December 05, 2014
Accepted: February 04, 2015

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ALUMINUM-INDUCED CASPASE-LIKE ACTIVITIES IN SOME GRAMINEAE SPECIES

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ABSTRACT

Aluminum toxicity is one of the major constraints for plant development and crop production on 67% of the total acidic soil area in the world. Al inhibits root growth and development leading to programmed cell death. In the present study, aluminum-induced caspase-like activities which control programmed cell death were investigated in four agronomic plant roots: *Secale cereale* (rye), *Triticosecale wittmack* (triticale), *Hordeum vulgare* (barley) and *Avena sativa* (oat). The roots were exposed to 100 μM $AlCl_3$ solution (pH 4.5) for 8 h at room temperature. After $AlCl_3$ treatment, root tips were ground in liquid N_2 and extracted. For determination of caspase-3, caspase-8 and caspase-9 activity, Chemicon's Caspase Colorimetric Activity Assay Kit was used. According to the manufacturer's instructions, supernatants were incubated with p-NA labeled substrates DEVD, IETD and LEHD, respectively. The caspase-like activity was measured at 405 nm. The results demonstrated that caspase-like proteolytic activities increased progressively beginning from ½ h in the root cells of rye, triticale, barley and oat after aluminum treatment.

KEYWORDS: Aluminum, caspase-3, caspase-8, caspase-9, Gramineae, programmed cell death.

1. INTRODUCTION

Aluminum (Al) is one of the most prevalent minerals in the earth's crust, comprising approximately 7%. Although it exists as structural component of soil in the form of insoluble aluminosilicates or oxides, the complex Al becomes solubilized in soil water when the soil is more acidic than 5.0. The phytotoxic aluminum species are absorbed by plant roots [1, 2]. Soluble Al is highly toxic to root growth principally, and the common effects are thick and stubby roots [1, 3], inhibition of cell and root elongation [4, 5], increase in cell wall rigidity [6], callose formation [5], structural alterations in the cytoskeleton [1, 7], production of toxic oxygen-free radicals [8], and interaction with DNA [1]. Although there are numerous researches on cellular and molecular mechanism of Al toxicity, there are limited studies on programmed cell death induced by Al.

Programmed cell death (PCD) is a physiological cell death process, which appears during development or under environmental stress. This type of genetically controlled cell disassembly executes with caspases (cysteine-containing aspartate-specific proteases) in animal systems [9]. The caspase-mediated apoptotic pathway is highly conserved in animal cells but such a cell death cascade was not found in plants [10]. Although no functional homologs of animal caspases have been defined in plants, indirect evidences, suggesting the existence in plants of true caspase-like activity, have been detected in plants during development and stress application [9, 11, 12]. Multiple experiments revealed caspase-like activities recognized in plants during hypersensitive response (HR), heat shock and development [11-13]. In support of these caspase-like activities, several researchers reported that caspase inhibitors could reduce or block the PCD [11, 14, 15]. After completion of the *Arabidopsis* genome sequence, it has been observed that only a few plant genes have been defined as orthologues of mammalian genes related with apoptosis. However, increased caspase-like activities in dying plant cells has proposed presence of special plant proteases homologous to animal caspases [16].

The cereals, such as barley, wheat, oat, rye and triticale belonging to Gramineae (Poaceae), have been considered as a staple food of the world population, and cultivated approximately on half of the world's crop land [17, 18]. It has been reported that acidic soils cover 30–40% of arable lands, and more than 50% of potential arable lands are under risk [19]. Ciamporova [7] reported that acidic soils often contain toxic concentrations of Al ions (10-100 μM). Because of the increasing soil acidity risk, the cereals are faced to Al toxicity resulting in decreased crop yield.

The aim of this study was, therefore, to investigate the time-dependent caspase-3, -8, and -9 like activity induced by Al under acidic conditions in 4 agronomic plant roots (rye, triticale, barley and oat).

2. MATERIAL AND METHODS

The seeds of *Secale cereale* cv. Aslım (rye), *Triticosecale wittmack* cv. Mikham (triticale), *Hordeum vulgare* cv. Çetin (barley) and *Avena sativa* cv. Seydişehir

(oat) were provided from The Central Research Institute For Field Crops (Ankara, Turkey). Surface-sterilized seeds were germinated in Petri dishes on moistened filter paper for 48 h. According to former studies and our preliminary tests, germinated seeds, which reached 0.5–1 cm root elongation, were exposed to 100 µM $AlCl_3$ solution (pH 4.5) for different times (0, 1/2, 1, 2, 3, 4, 5, 6, 7, 8 h) at room temperature. After $AlCl_3$ treatment, root tips were ground in liquid N_2 and extracted by the method of Lombardi *et al*. [13] with an extraction buffer (50 mM HEPES-KOH - pH 7, 10% sucrose, 0.1% CHAPS, 5 mM DTT, and 1 mM EDTA). The samples were kept on ice for 10 min and centrifuged at 14000 rpm and 4 ºC for 10 min. Protein concentration was determined by a Qubit 2.0 fluorometer. For determination of caspase-3, caspase-8 and caspase-9 activity, Chemicon's Caspase Colorimetric Activity Assay Kit was used. According to manufacturer's instructions, equal amounts of protein extracts were incubated at 37 °C for 2 h with *p*-NA (*p*-nitroaniline)-labeled substrates DEVD, IETD and LEHD, respectively. The caspase-like activity was measured at 405 nm with a microtiter plate. Comparison of the absorbance of *p*-NA from an apoptotic sample with an un-induced control allows determination of the fold increase in caspase activity. Ten seeds were used for each experimental group. All experiments were repeated three times and results were evaluated statistically.

3. RESULTS AND DISCUSSION

Caspases are a family of genes important for preserving homeostasis through regulating programmed cell death. Animal caspases are characterized by a number of structural and functional properties. They are normally present in the cell as inactive pro-enzymes which are activated by proteolytic cleavage at certain aspartic acid residues in the substrate. Caspases have been sub-classified by their mechanism of activity as initiator caspases (caspase-8 and -9) and executioner caspases (caspase-3, -6 and -7). It has been reported that caspase-3, -8 and -9 are situated at crucial junctions, particularly in animal apoptosis pathway [10, 20]. While caspase-8 initiates apoptosis in response to extracellular cues delivered in the form of ligands binding to death receptors (extrinsic pathway), caspase-9 initiates apoptosis with release of cytochrome c from mitochondria (intrinsic pathway) [21, 22]. Moreover, caspase-3 is activated both by extrinsic and intrinsic pathways and interacts with caspase-8 and caspase-9. Caspase-3 is also responsible for chromatin condensation and DNA fragmentation which are typical hallmarks of apoptosis [23, 24].

It is well-known that there is no functional homolog of animal caspases defined in plants. However, true caspase-like activities have been detected in plants during development and stress application [9].

In this context, the effects of Al toxicity on the time-dependent caspase-like activity in 4 agronomic samples were investigated through application of caspase-3, -8 and -9 substrates. The results demonstrated that caspase-like proteolytic activities increased progressively beginning from ½ h in the root cells of *Avena sativa* (oat), *Hordeum vulgare* (barley), *Secale cereale* (rye) and *Triticosecale wittmack* (triticale), after Al treatment (Figs. 1-4). As evidenced by the results, caspase-8 like activities, which are inducer caspases of extrinsic apoptotic pathway in animals,

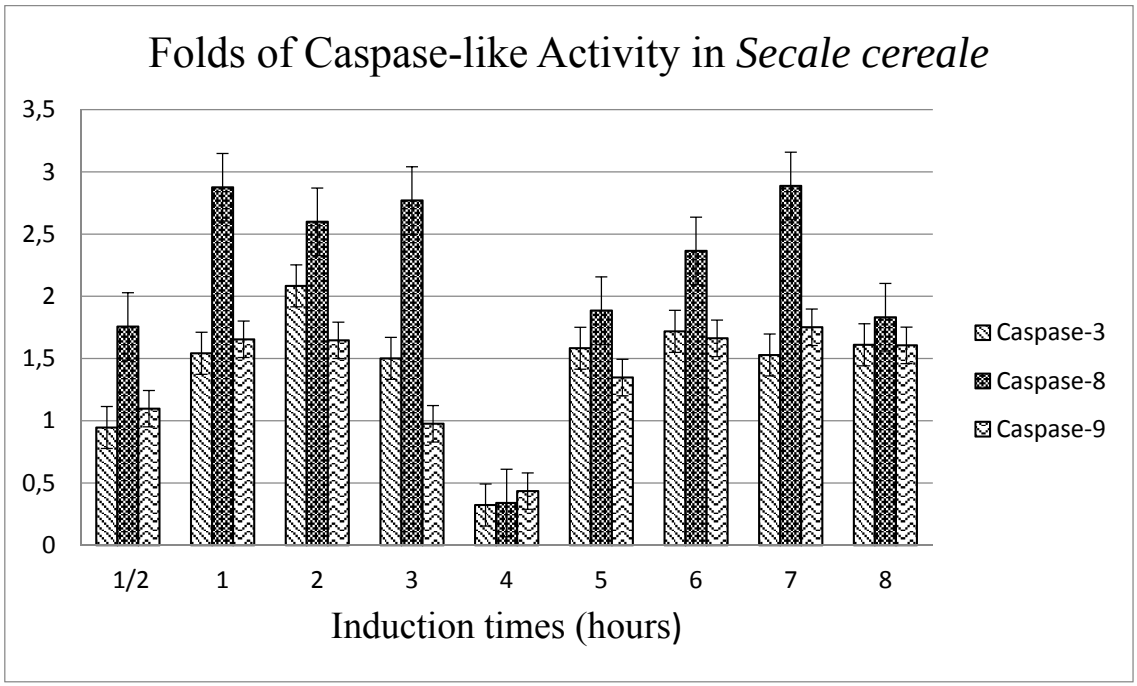

FIGURE 1 - The fold increase of caspase-3, -8 and -9 activities in *Secale cereale* with respect to control (mean ±SE).

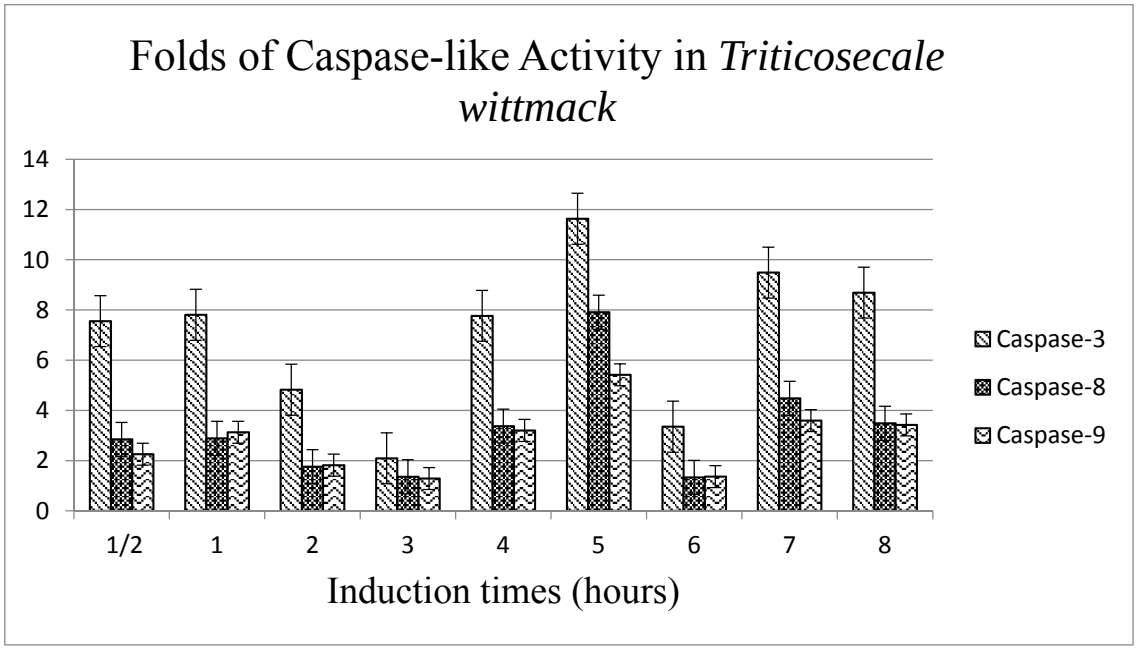

FIGURE 2 - The fold increase of caspase-3, -8 and -9 activities in *Triticosecale wittmack* with respect to control (mean ±SE).

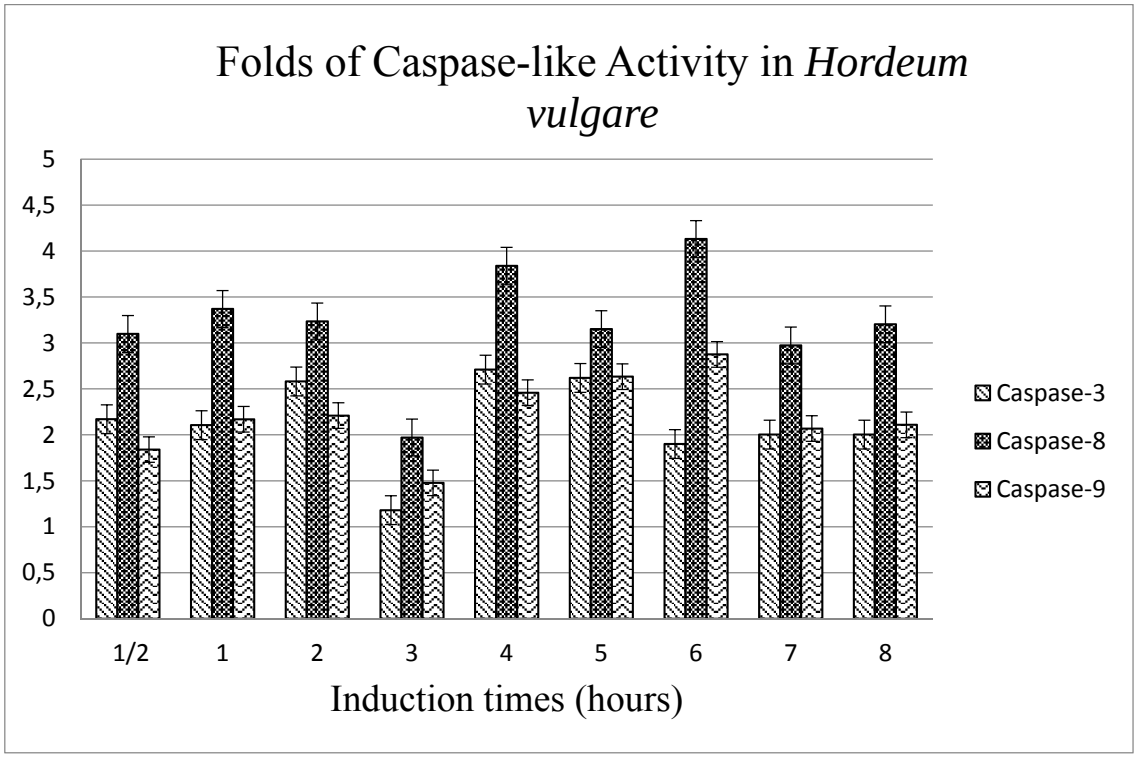

FIGURE 3 - The fold increase of caspase-3, -8 and -9 activities in *Hordeum vulgare* with respect to control (mean ±SE).

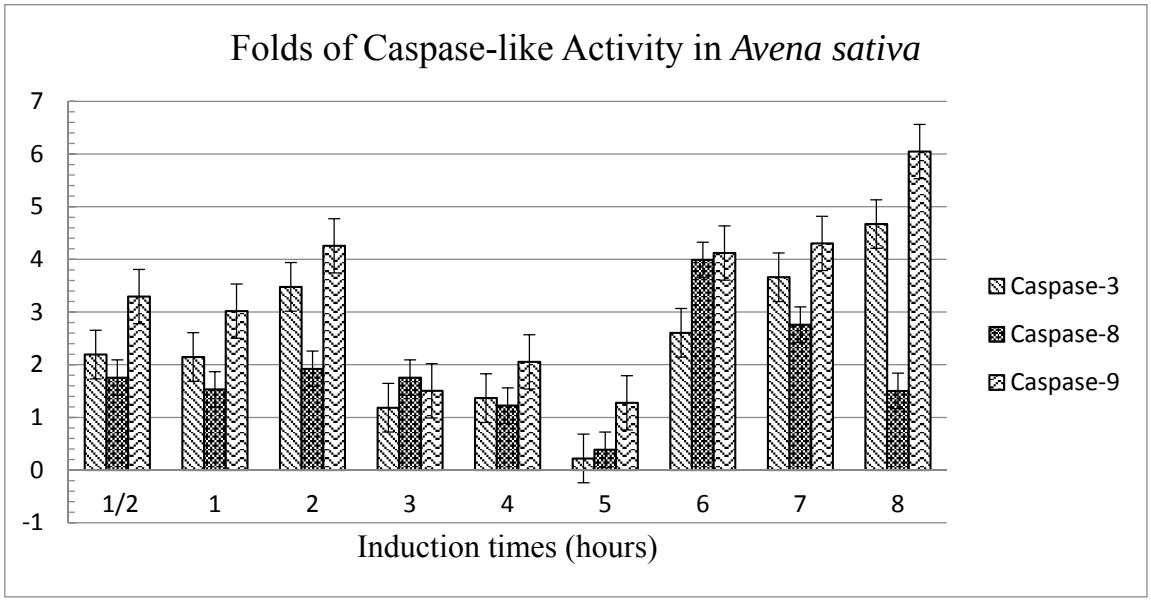

FIGURE 4 - The fold increase of caspase-3, -8 and -9 activities in *Avena sativa* with respect to control (mean ±SE).

were intensive in *S. cereale* and *H. vulgare* (Figs. 1, 3). Besides, caspase-3 like activities, which are responsible for chromatin condensation and DNA fragmentation, were dense in *T. wittmack* (Fig. 3). Moreover, caspase-9 like activities which are initiator caspases of mitochondrial (intrinsic) pathway, were observed in *A. sativa* (Fig. 4). It can be considered that Al toxicity induced programmed cell death triggering both intrinsic (mitochondrial) and extrinsic pathway. Both pathways activate caspase-3 like activities required for the typical hallmarks of apoptosis, and are crucial for apoptotic chromatin condensation and DNA fragmentation.

It has been reported that toxic Al forms affect the root growth and development within few min, after exposure to micro-molar concentrations [25, 26]. The presented results confirmed that Al induced caspase-like activities within ½ h after 100 μM Al exposure. To the best of our knowledge, there are limited studies which make contact between Al toxicity and caspase-like activity. Although the researchers mostly emphasized on mitochondria-dependent programmed cell death and caspase-3 like activity [27, 28] related to intrinsic pathway of cell death, the presented results comprising caspase-8 like activities, responsible for extrinsic apoptotic pathway under Al stress, will help advance understanding of Al toxicity and programmed cell death correlated to caspase-like activity.

4. CONCLUSIONS

Our results described that caspase-3, -8 and -9 like activities play an essential role in the regulatory program of PCD in the course of Al toxicity within ½ h in the roots of oat, barley, rye and triticale. This study will provide an understanding of the caspase-like activities that lead to programmed cell death in plants and improvement of crop products under Al stress.

ACKNOWLEDGEMENT

This study is supported by Research Foundation of Marmara University (BAPKO). project no. FEN-D-090414-0116

The authors have declared no conflict of interest.

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Received: December 11, 2014
Accepted: February 04, 2015

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FUNCTIONAL EDIBLE COATINGS AND FILMS FOR FRESH CUT FOOD PRODUCTS

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ABSTRACT

Food stuffs like fresh-cut fruits, vegetables, fish, meat and ready-to-eat meals which contain fresh produce are important sectors in food industry. Since these products usually undergo minimal processing and have fresh nature, they are very sensitive to quality degradation. Current strategies have tended to use cooling chain, modified atmosphere packaging, controlled atmosphere storage, or the application of preservatives. Even more, functional edible coating can be presented as a good carrier for some additives to the above-mentioned foods. Furthermore, edible packaging can serve as a promising approach for maintaining the safety and good quality of fresh-cut produces. Current research has shown that edible packaging with special properties could be a benefit for food preservation, and an advantageous way to decrease the amount of non-renewable packaging materials as a waste in the environment.

KEYWORDS:
coatings, edible, films, fresh-cut, functional

1. INTRODUCTION

Appearance and freshness of fresh-cut foods is an important factor that affects their marketing sector [1]. As edible coatings have a high capacity to carry additives, such as anti-browning agents, flavors, nutrients, spices and anti-microbial compounds, they can be a benefit to extend product shelf-life and reduce the risk of microbial spoilage [2].

Edible coating can be an alternative to synthetic packaging for fresh-cut and minimally processed products. Degradation of the foods that is caused by moisture and solution migration, gas exchange, respiration and oxidative reactions as well as physiological disorders can be reduced by the semi-permeable barrier of edible coatings [3].

Coatings are either applied to or formed directly on foods, while films, on the other hand, are self-supporting structures that can be used to wrap food products. They are located either on the food surface or as thin layers between different components of a food product. An example of the latter would be a film placed between the fruit and the dough in a pie to provide protection against transfer and mechanical stress [3].

When a film is used in composite form, it can utilize different characteristics of its components to have a special barrier and mechanical properties. The most appropriate substances for composite films are cellulose ethers, but also pectinates, chitosan, starch, alginates and carrageenans have been used in other composite films. These substances can be combined with stearic or palmitic acid, beeswax, acetylated monoglycerides and lecithins to form a useful film for food products [4].

Edible coating can be applied to improve the quality of fresh, frozen or processed meat, poultry and seafood products by reducing moisture loss, lowering lipid oxidation rate and discoloration, improving product appearance by omitting dripping, holding volatile flavors, carrying additives, such as antioxidant and antimicrobial agents, and decreasing oil uptake during frying [5]. The coating can hold a high concentration of the preservatives on the surface of the food [6].

Particularly, some potential benefits for meat products by edible coatings are as follows:

a) Moisture loss during storage of fresh or frozen meats leading to texture, flavor, and color changes, while also reducing saleable weight. For example, when meat is removed from vacuum packages, a 3–5% reduction in weight occurs due to moisture evaporation. Application of coatings prior to vacuum packaging could prevent this moisture loss, thereby having a significant economical impact by increasing saleable weight of products.

b) When fresh meat, poultry, or fish cuts are packaged in retail plastic trays, dripping of product juices occurs making such packages unattractive to consumers. Edible coatings could hold back juices, prevent dripping, enhance product presentation, and eliminate the need for placing absorbent pads at the bottom of trays.

c) The rates of rancidity causing lipid oxidation and brown coloration causing myoglobin oxidation in meats could be reduced by using edible coatings of low oxygen permeability, although not so low as to create anaerobic conditions.

d) Coatings applied on the surface of fish, poultry and meat pieces prior to battering, breading, and frying, could improve the products' nutritional value by reducing oil uptake during frying.

In this sense, the main goal of this article is to review and update the information available for the use of edible coatings as carriers of food ingredients (antimicrobials, anti-brownings, texture enhancers, nutraceuticals) to improve the safety, quality and functionality of fresh foods.

2. ANTIBROWNING

Operation/processing of fresh cut food can cause undesirable changes in appearance of the products during storage and marketing. The phenomenon is the result of converting phenolic compounds into dark components usually caused by polyphenoloxidase (PPO). Controlling of the colour change of freshly cut fruits has been done by dipping into antioxidant compounds [1]. One of the most extensively used additives that are applied to prevent fresh-cut fruit browning is ascorbic acid [7]. However, it is oxidized to dehydroascorbic acid; therefore, thiol-containing compounds, such as cysteine, N-acetylcysteine and reduced glutathione, have been used as alternatives to ascorbic acid. Even more, carboxylic acids (citric and oxalic acid) have been also suggested as effective inhibitors of enzyme browning of fresh-cut fruits [1]. For the purpose of applying anti-browning agents in edible coatings, alginates and gellan gum have been proven to be good carriers [3]. In order to reduce the oxidative reactions, degradation, or enzymatic browning of fruits and vegetables, several compounds, such as ascorbic acid, 4-hexyl resorcinol and some sulfur-containing amino acids or tripeptides (cysteine, glutathione) have been used as sulfite substitutes to improve shelf-life of minimally processed fruits [8].

3. TEXTURE ENHANCERS

Dramatic disorders of firmness can happen in fruit tissues by the action of pectic enzymes during operation. Enzymes and substrates are normally separated in sub-cellular compartmentalization which will be disrupted by cutting fruit tissues and can induce enzymatic reactions. One of the approaches for reducing loss of firmness is adding calcium salts to interact with pectic polymers and to form a cross-linked network that may improve mechanical strength and delay senescence. The other way that calcium ions can affect firmness of food texture is helping carbohydrates to form the well-known egg box model during gelation. This conformation is based on dimerization and aggregation of these dimers [9].

The film can carry components, such as flavoring, coloring and sweetening ones, to enhance the organoleptic properties of foods. Even more, small portions of foods (pear, bean and strawberry) not packaged individually can be covered by edible films to preserve their quality and safety [10].

Film properties, however, are important factors which can impact packaged foods appearance. For example, mechanical properties of protein-based films have shown to be better than carbohydrate or lipid-based films because proteins have a special structure (20 different monomers) leading to more intermolecular binding potential and variety range of functional properties. For instance, homogenous corn and wheat protein films were found to have less permeability against gases like oxygen or carbon dioxide but high water vapor permeability [11].

Alkaline treatment improves soy protein films appearance by making them clearer and more uniform with less bubbles but it does not affect barrier properties, such as water vapor, oxygen or carbon dioxide permeability. Some of the chemical agents, like glyceraldehyde, formaldehyde or glyoxal, can enhance cross-linking actions in protein films [10].

Results have shown that also increasing pH can improve tensile strength and elongation of the films but it may decrease lightness and barrier properties [12].

One of the other factors that may affect organoleptic properties of the films is the kind of plasticizer and its amount. For instance, films plasticized with glycerin may show an oily surface because of plasticizer migration [13].

Using glycerol as the plasticizer of the film may reduce water vapor resistance of the films but it can help to form more flexible film with higher elongation at break and lower tensile strength. The color evaluation had shown that increasing glycerol amount in a film caused L and b values increased while ΔE value decreased [14]. Glycerol has a hydrophilic nature and, if it is used at proper levels with regard to the kind of polymer, it may improve mechanical properties by increasing polymer chains mobility and decreasing intermolecular forces [14].

Straight-chain carbohydrates like amylase can form adjacent chains during processing resulting in a close matrix by inter-linkage and flexible chains. For methylcellulose, the chains remaining as bundle in native state, open up during gelatinization, and ultimately remain associated by both hydrophobic force among the methyl groups and hydrophilic force among the un-substituted regions. This produces a steady matrix with desired properties. Starch is one of the important components for making edible films consisting of amylase and amylopectin. Amylase is a linear chain carbohydrate but because of side chains of amylopectin matrix development is affected, and it leads to form a high absorbing capacity and poor mechanical properties. Starch is a preferred raw material for edible films because of its low cost and renewable nature [15]. One of the approaches to modify starch to form films with high tensile strength and satisfactory elongation value is to produce oxidized starch. Oxidation process causes a decrease in lipids, proteins and amylose [16].

4. NUTRACEUTICALS

Few studies have been done to incorporate nutraceutical compounds into edible films. Some researchers have estimated the effects of adding active compounds in the functionality of edible films. For instance, Mei and Zhao [17] evaluated the feasibility of milk protein-based edible films to carry high concentrations of calcium (5 or 10% w v^{-1}) and vitamin E (0.1 or 0.2% w v^{-1}). The water barrier property of the chitosan-based films was improved by increasing the concentration of mineral (5-20% w v^{-1} zinc lactate) or vitamin E in the film matrix. Addition of ascorbic acid (1% w v^{-1}) to the alginate and gellan-based edible coatings helped to preserve the natural ascorbic acid content in fresh-cut papaya, thus helping to maintain its nutritional quality throughout storage.

5. O_2 AND CO_2 BARRIERS

Generally, hydrophilic films like proteins and carbohydrates are good barriers to oxygen and carbon dioxide but poor ones to water vapor. However, films that are used for fruit and vegetable packaging should be moderate barriers to oxygen and carbon dioxide in order to avoid anaerobic respiration of living tissues [3]. Several edible coatings like cellulose, zein, soy protein and chitosan, because of being odorless, tasteless and transparent, are suitable for fruits and vegetables [18]. Oxygen permeability of most edible coatings was lower than the conventional plastics [19, 20]. CO_2 to O_2 permeability ratios of edible coatings and films are higher than those of plastic films. The permeability ratios of protein films are lower than those of cellulose films. These films have impressive gas barrier properties compared with those prepared from lipids and polysaccharides [10].

The barrier properties of proteins to gases are appropriate only in low and intermediate humidity (RH). Low resistance to gases and water vapor at high humidity is because of the hydrophilic nature of proteins.

6. WATER VAPOR PERMEABILITY

Plastic is the most used packaging material, but it causes the water vapor of the inner food to be condensed, and makes a potential point for microbial growth. So, the poor water vapor resistance of edible coatings and films would be a benefit to allow the water vapor cross the films and preventing its condensation. However, excessive water vapor permeability would be undesirable because it may cause extreme loss of water of fruits and vegetables [18].

Edible coatings of varying composition were applied on fresh apricots and green peppers. The water and vitamin C losses of these coated fresh foods were compared with those of uncoated ones. The main components of the coating were methyl cellulose (MC) and polyethylene glycol (PEG). Stearic acid (SA) and ascorbic acid (AA) or citric acid (CA) was added to the coating formulation to control the barrier properties toward water and oxygen. It was found that coatings of any composition studied lower the water loss rate of fresh apricots and green peppers [21].

Applying lipids in films and coatings can improve their barrier properties to water vapor. Garcia *et al.* [8] used sunflower oil in edible starch-based films and coatings intended for strawberries.

The poor water vapor resistance of protein films limits their application for food packaging. In many foods, especially low-moisture foods, lower water vapor permeability is preferable since low level water activity should be maintained to prevent deterioration due to enzymatic and chemical activities. Thus, lipids can be incorporated in the film formulation because of their hydrophobic nature; especially high melting point lipids, such as beeswax or carnauba wax, are appropriate for this point. Composite protein-lipid films had lower water vapor permeability values than control protein films from caseinates, whey protein, zein, and wheat gluten. The reduced migration of moisture in these kinds of films has also been studied [10]. Scientists reported that modification like denaturing of proteins forms films more resistant to water vapor but less transparent and flexible [2].

Loss of quality in products such as peeled carrots, which is a lightly processed and ready-to-eat product, may be exacerbated by surface dehydration and creating white blush on the surface of the product. Edible coatings consisting of emulsions incorporating caseinate with beeswax, stearic acid, or acetylated monoglyceride were applied to increased water vapor resistance and maintain good appearance [22].

Not only plasticizers increase the permeability of the films to oxygen and water vapor [3], but also other additives, such as anti-browning agents, which may enhance water loss through films and coatings [23].

One of the reasons for reverse effects of plasticizers on barrier properties of films is their hydrophilic nature which causes increasing the overall hydrophilic group density of the films [13]. Alginate (2%, w/v) and gellan (0.5%, w/v)-based edible coatings were formulated to study the effect of glycerol (G) and anti-browning agents (n-acetylcysteine and glutathione) on water vapor resistance (WVR). The WVR increased significantly from 15.70 and 14.60 s cm^{-1} to 19.2 and 27.6 s cm^{-1} for alginate and gellan coatings with sunflower oil, respectively, in comparison with oil-free coatings. The addition of sunflower oil in gellan was more effective than in alginate to increase the WVR of coated apples [3].

7. ANTIMICROBIAL AGENTS

Wounding of the tissue of fresh-cut foods during cutting makes them more perishable than their uncut commodities. One of the traditional ways to prevent microbial spoil-

age of these products is to dip them into aqueous solutions containing antimicrobial agents. However, this approach has some limitations because some of the microbial agents rapidly become neutralized or diffuse in to the food product; thus, the effects of active agents are limited [3]. In this sense, antimicrobial edible films can maintain high concentrations of antimicrobial agents on the surface of the food and provide increased inhibitory effects against spoilage and pathogenic bacteria. There are several categories of antimicrobials that can be potentially incorporated into edible films and coatings, including organic acids (acetic, benzoic, lactic, propionic, sorbic), fatty acid esters (glycerylmonolaurate), polypeptides (lysozyme, peroxidase, lactoferrin, nisin), plant essential oils (EOs) (cinnamon, oregano, lemongrass), nitrites and sulphites, among others [24]. In addition, spice extracts have been introduced for their ability to control meat spoilage.

However, nowadays, consumers prefer to have natural additives but not synthesized ones in their foods. Essential oils (EOs) outstand as an alternative to chemical preservatives, and their use in foods meets the demands of consumers for natural products, as reviewed by Rojas *et al.* [1]. Essential oils can cross through membranes and enter mitochondria because of their hydrophobicity; therefore, they can disturb the cell functionality and make it more permeable [24]. Factors that should be considered for the selection of a special antimicrobial agent for a particular food include properties of the food, the coating, and the effectiveness of the antimicrobial agents incorporated into the coating. In this sense, the effectiveness of an antimicrobial agent in an edible film must be approved before applying it on the food surface.

An antibacterial alginate-based edible film has been studied by incorporation of garlic oil as a natural antibacterial agent. Initially, 0.1% v/v garlic oil was tested in *in vitro* experiments against some food pathogenic bacteria. The presence of 0.1% v/v garlic oil in the nutrient broth decreased viable cell counts for *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus cereus* [25].

Applying antimicrobial agents in edible coatings and films is a kind of active packaging. Also, spice extracts have high antimicrobial activity, but their consumption in edible films and coatings is limited. Seydim *et al.* [6] studied antimicrobial properties of whey protein isolate (WPI) films containing 1.0–4.0% (w/v) ratios of oregano, rosemary and garlic essential oils ; they were tested against *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Lactobacillus plantarum*. The film containing oregano essential oil was the most effective against these bacteria at 2% level, with regard to those containing garlic and rosemary extracts ($P < 0.05$). The use of rosemary essential oil incorporated into WPI films did not exhibit any antimicrobial activity whereas that of the WPI film containing garlic essential oil was observed only at 3 and 4% level [6].

8. CONCLUSION

Different modification, such as enzyme or chemical reaction in films, can affect their permeability. For instance, incorporating anti-browning agents into edible films not only preserves the food from becoming brown during storage and can improve nutrient properties in the cases, such as using ascorbic acid as the active agents, but also it may affect the barrier and appearance properties, such as certain oxygen and carbon dioxide permeability values. Although protein films are good barriers against oxygen and carbon dioxide and have good mechanical properties, they are poor barriers against water vapor; thus, the incorporation of lipids greatly improves their water vapor barrier properties. Antimicrobial edible films and coatings may provide increased inhibitory effects against spoilage and pathogenic bacteria by maintaining effective concentrations of the active compounds on the food surfaces. In the case of using antimicrobial agent in edible packaging, essential oil is a good choice since the consumers' attitude is against applying chemical additives. Edible packaging can be one of the most popular technologies in food industry because more companies become aware of its benefit for both economical and quality aspects; additionally, more consumers accept the preference of this kind of packaging to traditional synthesized materials. This technology will likely become one of the most appropriate preservation methods for food products.

The authors have declared no conflict of interest.

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Received: August 31, 2014
Accepted: October 15, 2014

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AFS Book Reviews Bücherschau

Landwirtschaft für Quereinsteiger - Basics der Agrarwirtschaft

Christina Ziron/ Martin Ziron

1. Auflage 2015, **128** Seiten, kartoniert, zahlr. farb. Abb.; **ISBN 978-3-7690-2041-0**; € 7,50 (D) / € 7,70 (A) / sFr 12,00 (**Erhältlich** beim Verlag: DLG-Verlags-GmbH, Eschborner Landstraße 122, 60489 Frankfurt am Main, im Online Buchshop (www.dlg-verlag.de) sowie im Buchhandel

So können die Leser grundlegendes Basiswissen über Landwirtschaft für ihre tägliche Arbeit festigen, ihr Wissen in betriebswirtschaftlichen Themen landwirtschaftlicher Unternehmen vertiefen und den Landwirt in seiner Branche besser einordnen, dessen Arbeitsabläufe verstehen. Das ist für Mitarbeiter von Dienstleistern in der Agrarbranche (z.B. von Agenturen, Banken, Versicherern) ebenso wichtig wie für Führungs- und Managementkräfte aus Agribusiness-Unternehmen, die eine Ausbildung bzw. ein Studium in anderen Fächern absolviert haben und sich neu in der Branche etablieren. Mit dem Ratgeber erhalten Quereinsteiger und Interessierte die Möglichkeit, die Gespräche ihrer Fachkollegen zu bereichern, selbst mit Schlüsselkunden zu reden und ihre fachliche Expertise in den landwirtschaftlichen Zusammenhang einzuordnen.

Inalt: Betriebswirtschaftliche Entwicklung ● Pflanzenbau ● Tierhaltung ● Erneuerbare Energien ● Bodenbearbeitung und Bodenschutz ● Futterernte

Der Band erscheint in der Ratgeber-Reihe „**Agrar-Praxis kompakt**“ des DLG-Verlags: Praktisches Wissen – kurz, übersichtlich und einfach verständlich erklärt. Klein, praktisch, vielseitig, preisgünstig – die kleinen Helfer von Spezialisten für Praktiker.

Alles Wissenswerte rund um die Landwirtschaft von A wie „Abkalben“ bis Z wie „Zuckerrübe“ - „**Landwirtschaft für Quereinsteiger**“ aus dem DLG-Verlag bietet einen schnellen und kompakten Einblick in die Agrarwirtschaft und in die landwirtschaftliche Praxis.

Die Leser lernen die Einflüsse und Zusammenhänge der Subventions- und Haushaltspolitik sowie die Märkte kennen. Sie erfahren, wie das landwirtschaftliche Jahr von der Aussaat bis zur Ernte mit den verschiedenen Anbauverfahren funktioniert und wie die Tierzucht und -haltung aufgestellt sind. Die Betriebsformen in der Landwirtschaft werden ebenso vorgestellt wie die Unterschiede zwischen konventioneller und ökologischer Wirtschaftsweise in Pflanzenbau und Tierhaltung.

Chemistry and the Sense of Smell

Charles S. Sell (Ed.)

480 pages; tables, numerous pictures and photos (partly coloured); **ISBN: 978-0-470-55130-1**; May **201**4; Hardcover **135.00 EURO**; also available as e-book in all known E-book shops (All book prices inclusive VAT. Delivery to Germany, Austria, Switzerland, Liechtenstein and Luxembourg incur a fixed delivery charge of 3 Euros. Prices are subject to change without notice. The prices in euros stated here apply to Germany, Austria, Luxembourg, Switzerland and Liechtenstein. Orders from other countries will be forwarded to the relevant Wiley office. This may result in different prices and delivery charges.

A comprehensive overview of fragrance chemistry

Fragrance materials are universal, from personal care products to household cleaners, laundry products, and more. Although many of the scents themselves are synthesized in a lab, the actual mechanism of odour has long baffled chemists who attempt to model it for research. In *Chemistry and the Sense of Smell*, industry chemist Charles S. Sell explores the chemistry and biology surrounding the human detection and processing of odour, providing a comprehensive, single-volume guide to the totality of fragrance chemistry.

The correlation between molecular structure and odour is much more complex than initially thought, and the intricacies of the mechanism by which the brain interprets scent signals leaves much to be discovered. This book provides a solid foundation of fragrance chemistry and highlights the relationship between research and industry with topics such as:

- The analysis and characterization of odour
- The role scent plays in our lives
- The design and manufacture of new fragrance ingredients
- The relationship between molecular structure and odour
- The mechanism of olfaction
- Intellectual challenges and the future of the field

Complete with illustrations that clarify difficult concepts and the structures of the molecules under discussion, *Chemistry and the Sense of Smell* is an all-inclusive guide to the science of scent. For professionals in the fragrance industry or related fields, this book is one resource that should not be overlooked.

Therefore, it is warmly recommended to all interested readers, especially those engaged in scientific fields reaching from agriculture to veterinary medicine, like mentioned in the above section.

Table of Contents: Preface vii; Acknowledgments ix; Introduction 1; 1 Why Do We Have a Sense of Smell? 4; 2 The Mechanism of Olfaction 32; 3 Analysis and Characterisation of Odour 188; 4 The Sense of Smell in Our Lives 209; 5 The Scents of Nature 237; 6 Manufacture of Fragrance Ingredients 296; 7 The Design of New Fragrance Ingredients 357; 8 The Relationship Between Molecular Structure and Odour 388; 9 Intellectual Challenges in Fragrance Chemistry and the Future 420; Glossary 428. Index 437.

Author Information: CHARLES S. SELL (PHD) has recently retired from Givaudan after thirty-six years in the fragrance industry. His work has covered all aspects of synthetic organic chemistry as applied to the fragrance industry, from discovery of novel materials through process R&D to improvement of established chemical manufacturing processes. His interest in the design of novel fragrance ingredients, coupled with a long-standing interest in the chemistry of living organisms, led to a deepened investigation into olfaction.

AFS Press Release

TÜV SÜD explains process maturity according to the new ISO 9001

Munich. The final version of the revised standard for quality management systems is scheduled for release by the International Organisation for Standardisation (ISO) in autumn 2015. Many organisations have already started to address the expected changes, which are aimed at strengthening the benefits of quality management. To optimise their own processes they need to determine their status quo and identify approaches for introducing improvement. TÜV SÜD's experts provide information about how organisations can identify the maturity of their processes and initiate changes.

The revised ISO 9001 offers more flexibility regarding process documentation as a part of the management system. This applies to the documentation of management, strategy and core processes as well as support processes such as maintenance and qualification of persons. For very simple processes, for example, clear verbal instructions in conjunction with training material may be sufficient. Generally, however, traceable documentation in forms such as a checklist or an electronic workflow will be required to provide guidance for various work steps. To safeguard a high level of process quality, the new ISO 9001 includes an ambitious eight-step definition of the expected degree of process maturity. The relevant quality characteristics should be available and effective at each of these eight steps to ensure that organisations implement the standard successfully and realise the benefits offered by the new standard.

"At steps one and two, organisations define the inputs required and the outputs expected from their processes and the sequence and interactions of these processes in a form such as a process map or individual process sheets," says Ulrich Wegner, Head of the Certification Body of TÜV SÜD Management Service GmbH.

"At step 3, the organisation also review the responsibilities and authorities for these processes, using tools like responsibility matrices." Once this is completed, at step four organisations are expected to identify the technological and human resources needed. "Important aspects in this context include personnel and investment planning to respond appropriately to cases such as evident fluctuations or planned market expansion", adds Wegner.

At step five of process maturity, the focus is on risk-based thinking. To this end, organisations verify that they have identified all risks relevant for the key processes. Beyond the financial risks already analysed by many companies, there are other risks including the availability of expertise among knowledge owners and specialists, or market risks caused by innovative competitors. A quality management system according to the new ISO standard identifies these risks in direct association with the processes established by an organisation. While the old ISO 9001 focused predominantly on the avoidance of risks through appropriate preventive actions, the revised version also considers chances. To identify these chances, the detail-oriented process focus should be expanded to view the whole picture, particularly including customers' expectations and the context of the organisation.

Effective process operation and control are ensured at step six. This requires organisations to have either clear instructions for simple tasks in the service sector or process-integrated solutions, e.g. an ERP system, for complex production operations. Depending on the complexity involved, this step requires fast feedback or control loops and adequate communication. At step seven, the processes are evaluated with the help of appropriate monitoring and measurement methods. These are required in cases in which technical or personnel risks or instabilities were identified and, in particular, in cases involving risks related to customer requirements. Checking at an early stage for possible signs of non-conformities with the target is recommended. After all, a high level of accuracy in measurement results is of little use if the results are available too late. At step 8, the data gained from process evaluation enable management to make a robust decision regarding the necessary improvement actions. As outlined above, the process approach of the new ISO 9001 ensures a high level of transparency and thus supports organisations in the targeted triggering and driving of the improvement process.

Further information can be found at www.tuev-sued.de/management-systeme/iso-9001.

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