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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Optimization of *in vitro* multiplication for exotic banana (*Musa* spp.) in Pakistan

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The present attempt aimed to optimize micropropagation protocols supplemented with different concentrations and combinations of benzylaminopurine (BAP) (0, 2, 4, 6 mg L^{-1}) and indole acetic acid (IAA) (0.5 and 1.0 mg L^{-1}). Exotic banana (*Musa* spp) genotypes GCTCV-215 (AAA), 'Yangambi' Yangambi Km-5 (AAA) and FHIA-23 (AAAA) were used in research work. Experiments were conducted at Plant Tissue Culture Laboratory of Nuclear Institute of Agriculture (NIA), Tando Jam. Data collected for *in vitro* shoot consists of the following parameters: days for bud initiation, rate of shoot proliferation (%), number of multiple shoots, shoot length (cm) and fresh mass of shoot (g). Significant (p≤ 0.05) variations were observed for varieties, treatments and varieties x treatment for all the parameters. Synergistic effects of BAP and IAA were observed in GCTCV-215 and Yangambi Km-5. Out of various treatments, best concentration for multiple shoot in short period of time for GCTCV-215 and Yangambi Km-5 was found in 4.0 mg /l BAP + 0.5 mg L^{-1} IAA. Maximum fresh mass of shoot observed at same concentration and combination of BAP and IAA and for shoot length combination of 4.0 mg L^{-1} BAP with 1.0 mg L^{-1} IAA was found to be most suitable for GCTCV-215 and Yangambi Km-5. FHIA-23, show better performance in MS medium supplemented with only BAP at concentration 4.0 mg/L^{-1}. After development of root, *in vitro* plantlets were shifted from growth room to green house in polythene bags containing garden soil and humus mixture in ratio (1:1).

Key words: Micropropagation efficiency, exotic *musa* genotype, growth regulators.

INTRODUCTION

Bananas are large perennial herb (*Musa* spp.) belonging to the monocotyledonous family Musaceae. Banana is an important and widely grown fruit crop in the tropical and subtropical regions of the world (Darvari et al., 2010; Rahman et al., 2013). Banana serves as a source of instant energy and has lots of health benefits. In Pakistan, during the last five years banana has been grown on approximately 30 m ha with annual average production of 137 thousand tons (FAOSTAT, 2014). Due to popularity of the banana fruits for their nutritional properties the demand is increasing continuously and hence production of healthy planting material is necessary (Al-Amin et al., 2009). But because of the cultivation of susceptible cultivars, low soil fertility, higher wind velocity, drought stress and plants diseases, the banana production in Pakistan has reduced enormously. Genetic improvement

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in banana through conventionally approaches is restricted mainly due to reasons like variable polyploidy level, seedlessness, low female fertility and rising of asexual progeny with desirable characters (Bidabadi et al., 2012; Devendrakumar et al., 2013). The long term option for increasing banana yield could be the use of resistant varieties. Their multiplication through *in vitro* means could help to reduce the spread of pathogen to newer areas (Arvanitoyannis et al., 2008; Babita et al., 2013; Waman et al., 2014).

Success in *in vitro* multiplication is based on the growth and differentiation of plant tissues, which is viable only by the addition of suitable growth regulators (Gaspar et al., 2003). In shoot tip culture, cytokinins are known to enhance buds growth and shoot formation, while auxins promote root induction and development (North et al., 2012; Ngomuo et al., 2014). Benzylaminopurine (BAP) combined with auxins (indole acetic acid and naphthalene acetic acid) exhibit synergistic effect and hence has also been used by number of researchers (Al-Amin et al., 2009; Jafari et al., 2011; Sipen and Davey, 2012; Ngomuo et al., 2013). Tissue culture induced genetic variation in clonally propagated plant populations which may be genetic or epigenetic in nature; these variations are called 'somaclonal variation' (Larkin and Scowcroft, 1981; 1983). Such variation commonly occur in both *in vitro* and *in vivo* propagated *Musa* and had both positive and negative impact. Sometimes these variations create major obstacle for clonal uniformity (Nwauzoma and Jaja, 2013) but also exploited as source of genetic improvement of vegetatively propagated crops.

The present studies aimed at optimizing the kind and concentration of growth regulators for obtaining improved multiplications rate in high yielding genotypes of banana. This could help for area expansion of these superior exotic types in our country.

MATERIALS AND METHODS

Explants

For the establishment of *in vitro* shoot tip culture, suckers of three exotic genotypes of banana (*Musa* spp.) were selected from experimental field of Nuclear Institute of Agriculture (NIA), Tandojam, Pakistan. Of these two are triploid *viz*. 'Yangambi' Yangambi Km-5 (AAA) and Giant Cavendish Tissue Culture Variant GCTCV-215 (AAA), while Fundación Hondureña de Investigaciones Agrícolas FHIA-23 (AAAA) is a tetraploid.

Surface sterilization of explants

For decontamination, isolated explants from suckers were treated with 70% alcohol and 10% sodium hypochlorite separately for one and 20 min, respectively. After washing with sterile distilled water explants was trimmed to a size of about 6 to 8 mm.

Media and culture condition

Prepared explants were then cultured onto MS (Murashige and Skoog, 1962) basal medium with or without supplemental growth regulators. Culture medium for shoot induction and multiplication was prepared by supplemented with the MS medium with nine combinations of BAP with or without IAA (Table 1). The pH of medium was adjusted to 5.8 prior to placing in microwave oven. Prepared media were then poured into sterilized jars and autoclaved for 20 min at 121°C. For the establishment of culture all *in vitro* culture jars were transferred to growth room, at 25 ± 2°C temperature under 16/ 8 h light period provided by cool white florescent tubes with light intensity 2000 lux (27 µM m^{-2} s^{-1}).

Table 1. Different concentrations of BAP (with or without IAA) used during study.

Treatments	Concentrations (mg L^{-1})
MS Basal	0
BAP	2.0
BAP	4.0
BAP	6.0
BAP+IAA	2.0+0.5
BAP+IAA	2.0+1.0
BAP+IAA	4.0+0.5
BAP+IAA	4.0+1.0
BAP+IAA	6.0+0.5
BAP+IAA	6.0+1.0

Root induction media

For rooting, *in vitro* healthy shoots (of 4 to 5 cm) were transferred to half strength MS medium supplemented with 1.0 mg L^{-1} indole butyric acid (IBA).

Data collection and statistical analysis

Experiment was conducted in Completely Randomized Design (CRD) with four replications per treatment. Data were taken at 4 weeks intervals after subculture and was recorded every day for bud initiation per explant, proliferation rate (%), multiple shoots per explant, fresh mass (g) and shoots length (cm). Data statistically analyzed, was based on mean values per treatments and using analysis of variance (ANOVA). Statistical software STATISTIX (8.1version) was used.

RESULTS AND DISCUSSION

In the present work, effects of different BAP concentrations (2.0, 4.0 and 6.0 mg L^{-1}) with or without IAA (0.5 and 1.0 mg L^{-1}) were studies for optimizing the protocol for effective multiple shoot of the exotic *Musa* genotypes. The results are presented in Tables 2 to 4 and their analysis of variance is presented in Table 5. Result show significant ($p \leq 0.05$) differences for all studied parameters.

Effects of BAP and IAA on shoot proliferation and multiplication

Results indicate that in GCTCV-215 and Yangambi Km-5

Table 2. Effect of different concentrations of BAP and IAA on explant of GCTCV-215 in MS medium.

Treatments BAP+IAA mg L^{-1}	Explant/ Treatment	Variables				
		Rate of proliferation (%)	Average no of multiple shoot per explant	Days for bud initiation	Average fresh mass (g)	Average shoot length (cm)
0.0 + 0.0	10	13	1.25^{m-o}	17.0^{j}	2.12^{n-p}	2.00^{n}
2.0 + 0.0	10	25	2.50^{jk}	16.2^{jk}	2.80^{i-l}	2.56^{lm}
4.0 + 0.0	10	28	2.75^{ij}	14.0^{l}	3.00^{i-k}	3.25^{ij}
6.0 + 0.0	10	18	1.75^{lm}	14.5^{l}	2.75^{j-l}	3.83^{gh}
2.0 + 0.5	10	65	6.50^{c}	13.0^{m}	6.08^{c}	4.20^{ef}
2.0 + 1.0	10	75	7.50^{b}	11.2^{n}	7.32^{b}	4.99^{d}
4.0 + 0.5	10	88	8.75^{a}	7.0^{q}	8.77^{a}	5.16^{cd}
4.0 + 1.0	10	55	5.50^{d}	12.0^{n}	5.26^{e}	6.80^{a}
6.0 + 0.5	10	20	2.00^{kl}	18.0^{i}	3.63^{h}	3.18^{i-k}
6.0 + 1.0	10	18	1.75^{lm}	19.5^{gh}	3.18^{i}	2.05^{n}

Mean followed by dissimilar letters in a column are significantly different by least significant difference (LSD) test at P≤ 0.05.

Table 3. Effect of different concentrations of BAP and IAA on explant of Yangambi Km-5 in MS medium.

Treatments BAP+IAA mg L^{-1}	Explant/ Treatment	Variables				
		Rate of proliferation (%)	Average no of multiple shoots per explant	Days for bud initiation	Average fresh mass (g)	Average shoot length (cm)
0.0 + 0.0	10	10	1.00^{n-p}	28.0^{b}	2.25^{m-o}	1.65^{o}
2.0 + 0.0	10	20	2.00^{kl}	24.2^{d}	2.34^{m-o}	2.26^{mn}
4.0 + 0.0	10	25	2.50^{jk}	18.0^{i}	2.56^{lm}	3.06^{jk}
6.0 + 0.0	10	13	1.25^{m-o}	16.2^{jk}	2.00^{op}	2.87^{kl}
2.0 + 0.5	10	38	3.75^{fg}	15.5^{k}	4.02^{gh}	3.42^{i}
2.0 + 1.0	10	48	4.75^{e}	13.7^{lm}	4.75^{f}	4.00^{fg}
4.0 + 0.5	10	65	6.50^{c}	8.0^{p}	5.82^{cd}	5.50^{c}
4.0 + 1.0	10	35	3.50^{f-h}	$19.^{2gh}$	3.79^{gh}	6.11^{b}
6.0 + 0.5	10	18	1.75^{lm}	20.0^{g}	2.50^{l-n}	2.94^{jk}
6.0 + 1.0	10	10	1.00^{n-p}	22.7^{e}	3.06^{ij}	2.00^{n}

Mean followed by dissimilar letters in a column are significantly different by least significant difference (LSD) test at P≤ 0.05.

combination of BAP and IAA significantly (p≤ 0.05) induced bud proliferation and multiplication in short period of days (Table 2 and 4). However, in FHIA-23 BAP alone had significant effect on bud proliferation as well as on multiplication (Table 4). As compared to control, in GCTCV-215

Table 4. Effect of different concentrations of BAP and IAA on explant of FHIA-23 in MS medium.

Treatments BAP+IAA mg L-1	Explant/ Treatment	Variables				
		Rate of proliferation (%)	Average no of multiple shoot per explant	Days for bud Initiation	Average fresh mass (g)	Average shoot length (cm)
0.0 + 0.0	10	8	0.75^{op}	29.7^{a}	2.47^{l-n}	0.80^{p}
2.0 + 0.0	10	15	1.50^{l-n}	26.2^{c}	2.85^{i-l}	2.08^{n}
4.0 + 0.0	10	48	4.75^{e}	25.0^{d}	5.60^{de}	4.96^{d}
6.0 + 0.0	10	10	1.00^{n-p}	21.0^{f}	2.61^{k-m}	2.54^{lm}
2.0 + 0.5	10	33	3.25^{g-i}	19.0^{h}	4.08^{g}	3.00^{jk}
2.0 + 1.0	10	40	4.00^{f}	10.0^{o}	3.76^{gh}	4.40^{e}
4.0 + 0.5	10	30	3.00^{h-j}	16.3^{jk}	3.07^{ij}	4.01^{fg}
4.0 + 1.0	10	25	2.50^{jk}	22.7^{e}	2.81^{i-l}	3.49^{hi}
6.0 + 0.5	10	10	1.00^{n-p}	25.0^{d}	2.56^{lm}	2.16^{n}
6.0 + 1.0	10	5	0.5^{0p}	27.5^{b}	1.75^{p}	1.63^{o}

Mean followed by dissimilar letters in a column are significantly different by least significant difference (LSD) test at P≤ 0.05.

Table 5. Analysis of Variance (ANOVA) for days to bud initiation, number of shoots, fresh mass (g) and shoot length (cm)

Source of variation	Mean sum of squares of shoot parameters				
	DF	Days to bud initiation	Number of shoots	Fresh mass (g)	shoot length (cm)
Replications	3	0.343	0.7667	0.0608	0.0940
Varieties	2	642.187*	33.8083*	21.4006*	7.9918*
Treatments	9	273.056*	42.4778*	19.6365*	20.5552*
V x Treat	18	24.702*	5.0583*	5.8150*	2.0401*
Error	87	0.335	0.2149	0.0800	0.0612
Total	119	--	--	--	--

The results are for the mean of 4 replicate (Significant at P≤0.05 =*).

(8.75 shoots/ explant) and Yangambi Km-5 (6.50 shoots/ explant) the highest numbers of multiple shoots with high proliferation rate (87 and 65%), respectively, were observed in MS media concentrated with 4.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA while in FHIA-23, highest number of shoot multiplication (4.75 shoots/ explant) with high shoot proliferation rate (48%) was observed in medium supplemented with 4.0 mg L^{-1} BAP (Figure 1). Results indicate that media containing with high level of BAP alone or in combinations with IAA relatively decreased the number of shoot multiplication in all genotypes. Low concentration also showed slight increase in bud proliferation when compared with control (Figure 3). Concentration of BAP 4.0 mg L^{-1} along with IAA was found optimum for selected varieties of *musa.* The results agree with the findings of Muhammad et al. (2007). They found superior multiplication ratio at same concentration of 4.0 mg L^{-1} BAP along with 1.0 mg L^{-1} IAA. Habiba et al. (2002)

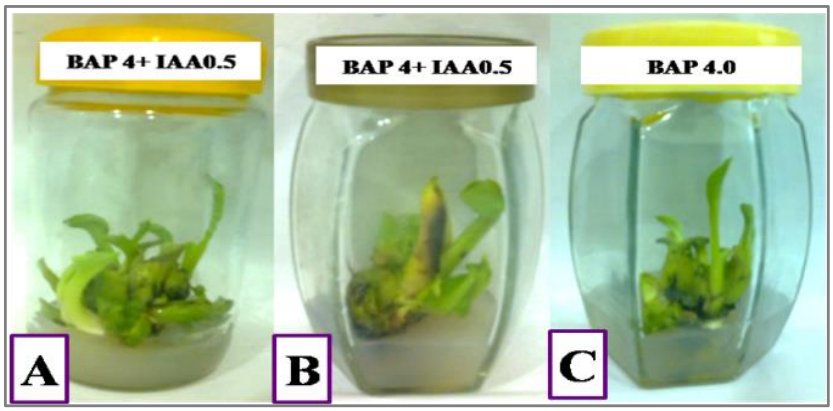

Figure 1. Multiple shoot produced by (A) GCTCV-215, (B) Yangambi Km-5 and (C) FHIA-23.

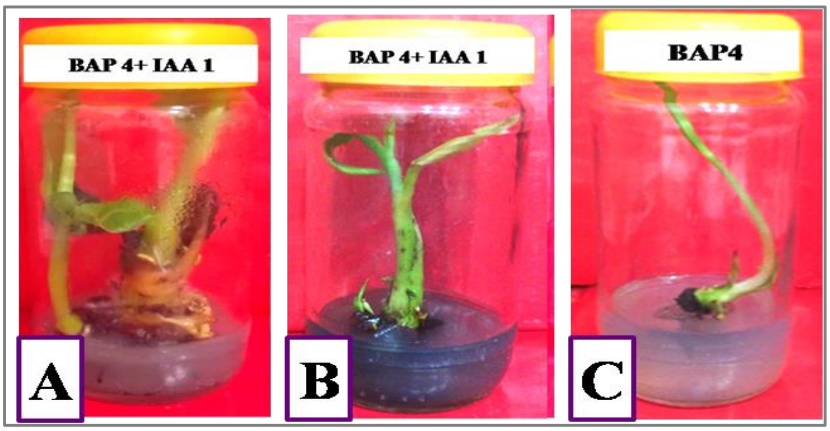

Figure 2. Variation in shoot length (A) GCTCV-215, (B) Yangambi Km-5 and (C) FHIA-23.

and Ahmed et al. (2014), also reported synergistic effect of BAP and IAA at nearly similar combination of 4.0 mg L^{-1} BAP and 2.0 mg L^{-1} IAA. Frequency of multiple shoot formation was 3 to 4 folds due to sub culture in the same fresh media. Current findings supported the earlier studies, which suggest that rate of shoot multiplication was dependent on specific genotype. Gubbuk and Pekmezcu (2004) and Ngomuo et al. (2013) suggested that apart from the genotypes behavior, shoot proliferation was also affected by exogenous cytokinin concentration in growth medium. Suitable cytokinins concentrations in medium inhabit apical dominancy and support initiation of lateral shoots (Jafari et al., 2011). Besides, the presence of exogenous phytohormones, *in vitro* organogenesis depends on the interaction of various factors associated with the endogenous phytohormones, their concentrations, and rate of metabolisms and presence of nutrients in medium (Skoog and Miller, 1957; Ammirato, 1986; Ahmed et al., 2014).

Effects of BAP and IAA on fresh mass and shoot length

Result shows that fresh mass significantly ($p \leq 0.05$) increased at 4.0 mg L^{-1} concentration of BAP along with 0.5 mg L^{-1} IAA in GCTCV-215 and Yangambi Km-5 (Table 3 and 4). Among nine treatments, as compared to control GCTCV-215 (8.77 g) ,Yangambi Km-5 (5.82 g) showed maximum fresh mass in MS medium supplemented with 4.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA. Whereas, in FHIA-23 (5.60 g), MS medium supplemented with 4.0 mg L^{-1} BAP alone gave good response (Figure 2). It was evaluated that hormonal response is specific genotypic dependent. In various treatments MS media supplemented with low concentration of BAP 2.0 and 4.0 mg/ L^{-1} along with IAA, was considered as optimal for production of maximum fresh mass for studied *Musa* genotypes. Quite similar synergistic effect of BAP and IAA on maximum regeneration of shoot at concentration 2.0 and 0.5 mg L^{-1},

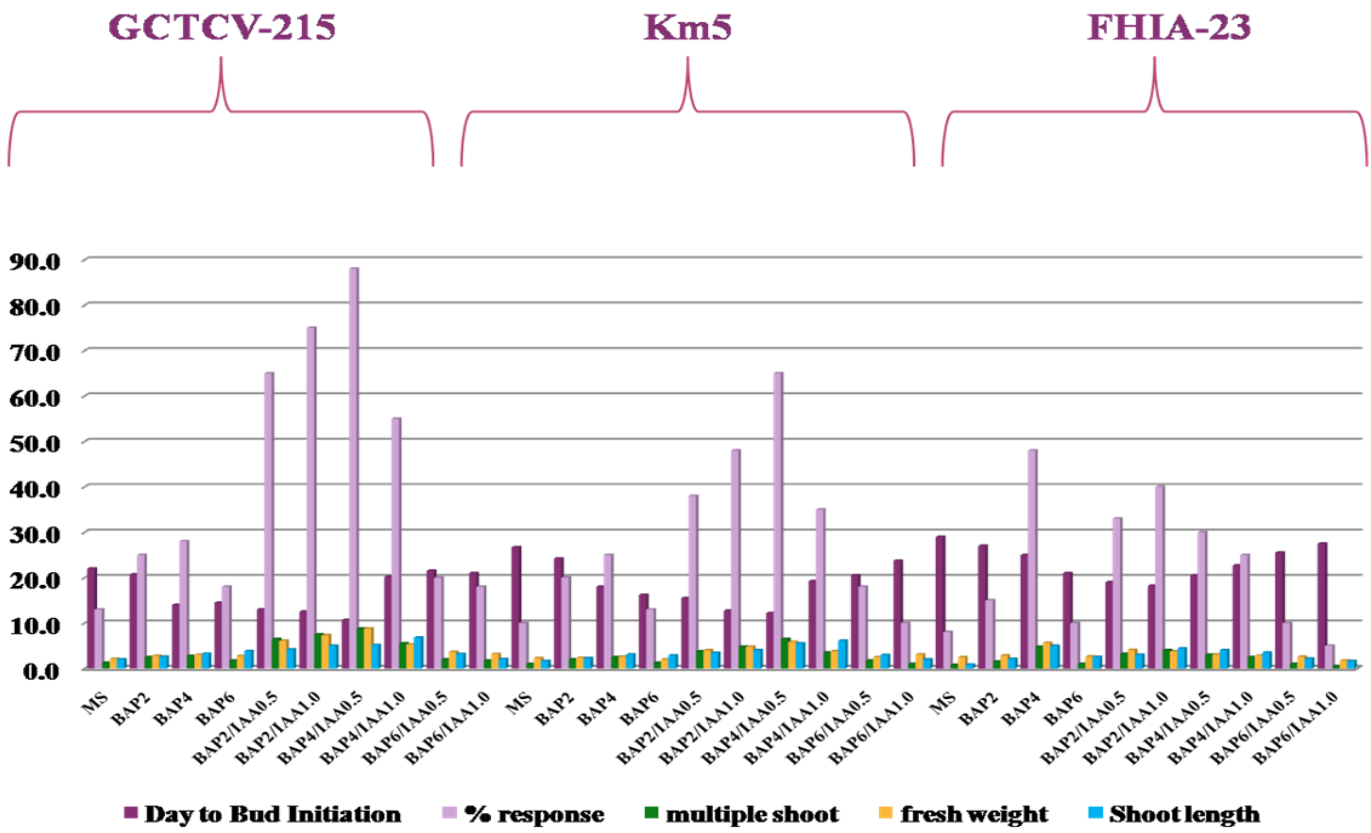

Figure 3. Effect of BAP and IAA on *in vitro* shoot tip culture of all genotypes.

respectively, was reported by Anbazhagan et al. (2014). In GCTCV-215 (6.80 cm) and 'Yangambi' Km-5 (6.11 cm), highest shoot length as compared to control was observed in medium concentrated with 4.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA, while in FHIA-23 (4.96 cm), highest shoot length was observed in 4.0 mg L^{-1} BAP alone (Figure 3). Results indicate that media supplemented with high level of BAP alone or in combinations with IAA relatively decreased the shoot length in all genotypes. In all genotypes, cytokinin (BAP) and auxin (IAA) showed an effective synergistic effect on shoot length. Jafari et al. (2011) and Dhed et al. (1991) reported that combination of BAP with IAA become more effective for shoot elongation. Other researchers reported nearly similar effect of BAP and IAA on shoot length (Iqbal et al., 2013; Rahaman et al., 2013; Ahmed et al., 2014). According to examined data for highest shoots length, concentration of BAP 4.0 mg/L^{-1} in interaction with 1.0 mg/L^{-1} IAA was suggested as optimal for significant shoot length.

Conclusion

Presence of BAP along with IAA in the culture medium induced efficient shoot multiplication in selected genotypes than BAP alone and the effect was genotype dependent. The optimum concentration of BAP for *in vitro* shoot multiplication of GCTCV-215, Yangambi Km-5 and FHIA-23 was 4.0 mg L^{-1} with 0.5 mg L^{-1} IAA. Hence, it could be used in future for *in vitro* propagation of these varieties of banana. It was also noticed that two genotypes GCTCV-215(AAA) and Yangambi (AAA) gave significant response in most defined media as compared to FHIA-23 (AAAA). Both belong to similar genomic constitution (triploid). So, maybe all genotypes behaved according to their ploidy level.

In future further study is required to carry out research work on the influence of ploidy level on micropropagation efficiency in Banana.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Growth performance and nutrient quality of three *Moringa oleifera* accessions grown as potplant under varied manure rates and watering intervals

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Growth performance and nutrient quality of three *Moringa oleifera* accessions grown as potherbs under varied manure rates and watering intervals were investigated at the Department of Crop Science, University of Nigeria, Nsukka. The treatments included three accessions of Moringa (Awo-Anaekpa, Idere and Kano), three pig manure rates (0, 10 and 20 t/ha) and three watering intervals (3, 4 and 5 days). These were laid out as 3 x 3 x 3 factorial in a completely randomized design, replicated three times. Results showed that seeds of Awo-anekpa accessions had the highest cumulative emergence percentage (97%) and number of emerged seedlings. Plant height and stem girth at 3 months, and number of leaves at 2 and 3 months, after treatment application were higher in Awo-anekpa and Idere accessions than in Kano accession. The leaf proximate composition showed that Idere accession accumulated the highest ash (7.6%) and crude fibre (11.9%). Awo-anekpa contained the highest carbohydrate (41.1%) and moisture (20.7%), while the highest crude protein (26.9%) and oil content (3.0%) were found in Kano accession. Generally, plant height, stem girth and number of leaves increased with increase in manure rate; however, the leaf proximate compositions were not significantly ($p > 0.05$) influenced by manure application. Only stem girth was significantly ($p < 0.05$) affected by watering interval. The thickest stem girth (12.2 mm) was associated with 3 day watering interval, whereas 5 day watering interval produced the tiniest stem girth (10.8 mm). Second order interaction of accession, manure rate and watering interval suggests that Idere accession grown with 20t/ha of pig manure and watered at 3 day interval was most adaptable to the container growing condition. Evidences from this study suggest that *Moringa* can successfully be grown as a potherb if appropriate horticultural practices are followed.

Key words: *Moringa oleifera*, potherb, pig manure, watering interval, leaf proximate qualities.

INTRODUCTION

Moringa oleifera is commonly known as drumstick tree, horseradish tree and ben oil tree. It is a multi-purpose plant widely known for its ethno-medicinal (Price, 2007; Mughal et al., 1999; Farooq et al., 2012) and culinary properties (Price, 2007; Farooq et al., 2012; Stevens et al., 2013). All parts of the Moringa tree are edible and have long been consumed by humans (Fahey, 2005). In developing countries, Moringa has the potential to improve

nutrition, boost food security, foster rural development, and support sustainable land care (NRC, 2006). *M. oleifera* leaves have been reported a valuable source of macro and micro nutrients, being a significant source of beta-carotene, vitamin C, protein, calcium, iron and potassium (Fuglie, 1999; Olugbemi et al., 2010). Moringa plant is a soft wood tree with low timber quality but has been reported to provide nutritional, medicinal and industrial uses to man, livestock feed and crop nutritional benefits (Fuglie, 1999). It is increasingly becoming popular for use as food supplements especially by nursing mothers, as a weaning food for children due to its nutritional benefits. It is reported to contain more than 92 useful compounds; including 46 antioxidants, 36 anti-inflammatory constituents, 18 amino acids and 9 essential amino acids (Duke, 1983; Olsen et al., 1987; Nnam, 2009). There is a recent upsurge in utilization of Moringa (Stevens et al., 2013) and research interest has grown tremendously in Nigeria. Most research efforts in Nigeria are on basic science, nutritional and medicinal properties and utilization of the plant (Nnam, 2009; Eze et al., 2012). Study on germplasm collection and evaluation are probably not adequately documented except the report by Ndubuaku et al. (2014). Besides, there is no information in relevant literature on growing Moringa as a potherb (container grown vegetable).

Developing horticultural techniques for growing Moringa plant as a potherb will ensure that urban dwellers could plant the crop within the house and compound with ease. More so, growing Moringa as a potherb makes it readily available to the household for consumption since the planting can be done on the balcony or backyard where there is sufficient insolation. A poor availability of essential nutrients in substrate for growing container plants reduces crop growth and yield (Fried and Broeshart, 1967; Baiyeri and Mbah, 2006). A vast array of organic wastes, compost and animal manures, as well as, inorganic fertilizers serve as amendments to improve on the soil/substrate fertility status (Stoffella et al., 1997). Just like other animal manures, pig dung serve as low cost fertilizer when applied to agricultural soils (Babalola and Adigun, 2013). Organic fertilizers supply plant nutrients in readily available form, and modify the physicochemical attributes of soils for improved crop performance (Aba et al., 2011); and pig manure is readily available in Nsukka environs owing to the prevalence of commercial pig farms in the area (Ezeibe, 2010).

Thus, this study was conducted to evaluate the growth performance and nutritional quality of three Nigerian accessions of Moringa grown as potherbs, using three rates of pig manure and three watering intervals. Water management in commercial nurseries, particularly with container grown plants could be tasking and expensive. Judicious management of irrigation water in terms of volume and/or frequency of application could help nurserymen cut cost, save labour, and maximize crop water use.

MATERIALS AND METHODS

Study area

This experiment was conducted at the Department of Crop Science Teaching and Research Farm, University of Nigeria, Nsukka. Nsukka lies between latitude 06° 52'N and longitude 07°24'E and at an altitude of 447 m above sea level. The average daily temperature ranges between 27 and 28°C, and two predominant seasons prevail – the rainy season lasting from April to October and the dry season from November to March (Ofomata, 1978). The vegetation of the area is derived savannah.

Experimental design and treatment

The experimental design was a 3×3×3 factorial in completely randomized design (CRD) making up 27 treatment combinations replicated three times in a pot experiment. The treatment combinations comprised three Nigerian accessions of Moringa (Awo-Anaekpa, Idere and Kano, respectively obtained from Kogi, Oyo and Kano states of Nigeria), three pig manure rates (0, 10 and 20 t/ha) and three watering intervals (3, 4 and 5 days). Both field and laboratory experiments were conducted.

Field experiment

The experiment was conducted using 18 L perforated nursery buckets filled with 17.6 kg topsoil (Figure 1). Substrates were moistened to container capacity and thereafter five seeds were sown per bucket. Watering was done twice daily, morning and evening due to low relative humidity accentuated by the dry harmattan season. Dry grass mulch was applied 3 days after planting to help conserve moisture and to enhance germination. Seedling emergence was noticed after 9 days. Seedlings were later thinned down to three per bucket. Weeds were handpicked when necessary. The application of manure and watering treatments commenced 8 weeks after seed planting. Manure was applied in three split doses at 2 week intervals. The first application was done at the 8th week after seeding; the second application was done at the 10th week, while the third application was at the 12th week. Watering was done at every 3, 4, or 5 days until the experiment was terminated (at the third month after treatment application). The irrigation treatment commenced with the application of water at 2.8 L per bucket, which was equivalent to the container capacity.

Field data collection

The following data were measured: percentage seedling emergence was calculated as the ratio of emerged seedlings to the total number of seeds sown multiplied by 100; mean daily emergence was calculated as the total number of emerged

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Figure 1. *Moringa oleifera* accessions growing in 18 L buckets.

Table 1. The effect of accession on *Moringa oleifera* seedling emergence parameters.

Accession	Cumulative emergence percentage	Emergence span	Mean daily emergence	Number of non-emerged seedlings	Number of emerged seedlings
Awo-anekpa	97.0	3.0	2.7	0.15	4.9
Idere	56.3	1.9	2.0	2.4	2.6
Kano	88.9	3.1	2.2	0.6	4.4
$LSD_{0.05}$	9.7	ns	ns	0.4	0.4

seedlings divided by the cumulative number of days to seedling emergence; emergence span was estimated as the number of days from first seedling emergence to the last seedling emergence. Plant growth parameters measured included: Plant height (cm) and number of leaflets per plant which were determined before manure and watering treatment application and were thereafter repeated on monthly basis for three consecutive months. The stem girth (mm) of each plant was measured at 20 weeks after planting (WAP). At the 21st week, destructive sampling of the plants was done and the leaves were dried at the Department of Crop Science glasshouse.

Laboratory analysis

The laboratory analysis was carried out at the Department of Animal Science Analytical Laboratory, University of Nigeria, Nsukka. Proximate qualities of the air-dried leaf samples were carried out to determine the crude protein, crude fibre, moisture, ash, fat and carbohydrate contents. These proximate qualities were determined using the standard procedures of Association of Official Analytical Chemistry (AOAC, 1990). The crude protein content of the samples was determined using the Khedahl technique (Pearson, 1976). The ash of an agricultural material is the inorganic or mineral residue remaining after the organic matter has been burnt away (Pearson, 1976), hence was determined by subtraction of all other component fractions from 100%.

Statistical analysis

All data collected were subjected to analysis of variance (ANOVA) following the three-way procedure for factorial experiments using GenStat Release 10.3 DE (2011). Significant treatment means were separated using Fisher's Least Significant Difference at 5% probability level.

RESULTS

Data in Table 1 shows the results of the seedling emergence parameters as influenced by accession. It was observed that emergence span and mean daily emergence were non-significant ($P > 0.05$) but the number of emerged seedlings, non-emerged seedlings and cumulative emergence percentage significantly differed with accessions. The cumulative emergence percentage (97%) and number of emerged seedlings (4.9) were highest in Awo-anekpa accession. Invariably, the number of non-emerged seedlings was least with Awo-anekpa (0.15). Comparatively, the Idere accession had the least number of emerged seedlings (2.6),

Table 2. Main effects of accession, manure and watering interval on the plant height and stem girth of *Moringa oleifera,* months after treatment application.

Treatment	Plant height (cm)				
	Initial	1 month	2 months	3 months	*Stem girth (mm)
Accession					
Awo-anekpa	27.1	51.1	64.3	86.5	12.5
Idere	25.8	48.2	64.8	86.4	12.7
Kano	24.5	45.3	60.6	77.3	9.5
$LSD_{0.05}$	ns	ns	ns	8.2	1.0
Manure (t/ha)					
0	27.7	36.3	40.7	56.8	9.0
10	24.3	48.3	64.3	83.2	12.1
20	25.4	60.0	84.7	110.2	14.0
$LSD_{0.05}$	ns	6.0	6.5	8.2	1.0
Watering interval					
3	26.1	50.0	66.4	86.5	12.2
4	25.8	49.4	64.6	84.6	11.9
5	25.5	45.3	58.7	79.1	10.8
$LSD_{0.05}$	ns	ns	ns	ns	ns

ns = non-significant; *Stem girth (mm) was determined 3 months after treatment application.

cumulative emergence percentage of 56.3% and consequently the highest number of non-emerged seedlings (2.4).

Main effects of accession, manure and watering interval on the plant height and stem girth of *M. oleifera*

Table 2 shows the main effects of accession, manure and watering interval on the plant height and stem girth of *M. oleifera*. The baseline data on plant height (that is, the plant height recorded before manure/irrigation treatment application) and the subsequent heights after one and two months of treatment application were not significantly ($p > 0.05$) different among the accessions. But at three months after treatment application, Awo-anekpa and Idere accessions were statistically taller and had thicker stem girth than *Kano* accession. The manure effect indicated that the plant heights at one, two and three months were significantly ($p \leq 0.05$) influenced by the manure rate (Table 2). At each month interval, plant height increased with increasing manure rate. The tallest plants were obtained with the application of 20 t/ha pig manure (that is, 60, 84.7 and 110.2 cm, respectively for one, two and three months after treatment application). A similar trend exists for stem girth. The no manure application gave the least values for plant height and stem girth. Watering intervals did not significantly ($p > 0.05$) affect the plant height at all the month intervals (Table 2). However, 3 days watering interval produced the widest stem girth (12.2 mm) while the 5 days interval had the least stem girth (10.8 mm).

Effect of accession, manure rate and watering interval on the number of leaves per plant

The effects of accession, manure and watering on the number of leaves per plant at different periods of growth are shown in Table 3. The highest number of leaves at 1 month after treatment application was associated with Idere while Kano and Awo-anekpa accessions had numerically similar number of leaves. At 2 and 3 months, Awo-anekpa and Idere accessions had the highest number of leaves. At 1 month after treatment application, there was no significant difference in number of leaves per plant among the manure rates (Table 3); but at 2 and 3 months, the number of leaves increased with increase in manure rate. The application of 20 t/ha pig manure produced the highest number of leaves in the 2 and 3 months after treatment application. Watering interval did not influence the number of leaves throughout the growth stages (Table 3). The interaction effect of accession and manure rate indicated that the number of leaves and stem girth increased with increase in manure rate irrespective of the accession (Table 4). The number of leaves and stem girth were highest at 20 t/ha manure rate for the three accessions. The least number of leaves and thinnest stem were obtained with no manure

Table 3. Main effects of accession, manure and watering interval on number of leaves per plant of *Moringa oleifera*, months after treatment application.

Treatment	Number of leaves			
	Initial	1 month	2 months	3 months
Accession				
Awo-anekpa	10.5	8.5	8.9	11.8
Idere	10.9	9.8	8.7	10.8
Kano	9.2	8.7	6.8	8.5
$LSD_{0.05}$	0.9	0.8	0.6	1.1
Manure (t/ha)				
0	9.7	8.5	6.9	9.4
10	10.3	9.4	8.4	10.4
20	10.5	9.0	9.1	11.4
$LSD_{0.05}$	ns	ns	0.6	1.1
Watering interval				
3	10.4	9.3	8.6	10.6
4	10.0	9.2	7.9	10.1
5	10.2	8.5	8.0	10.4
$LSD_{0.05}$	ns	ns	ns	ns

ns = non-significant

Table 4. The interaction effects of accession and manure rate on the number of *Moringa oleifera* leaves and stem girth, three months after treatment application.

Accession	Manure rate (t/ha)	Number of leaves	Stem girth (mm)
Awo-anekpa	0	8.3	10.1
	10	9.0	12.9
	20	9.4	14.7
Idere	0	6.8	9.4
	10	8.9	12.3
	20	10.6	16.6
Kano	0	5.7	7.3
	10	7.4	10.9
	20	7.6	10.5
$LSD_{0.05}$		1.2	1.8

application in Kano accession. The values for the number of leaves and stem girth in Kano accession were significantly ($p < 0.05$) smallest at all manure rates.

Effect of accession, manure rate and watering interval on the leaf proximate composition of *M. oleifera*

The leaf proximate composition was significantly influenced by accession (Table 5). Idere accession had the highest ash content (7.6%) and crude fibre (11.9%) while Kano accession had the least value (5.5%) for ash content. Awo-anekpa accession had the highest carbohydrate (41%) and moisture contents (20.7%), although the value for the moisture content was statistically similar with that of Idere (19.9%). The least carbohydrate and moisture contents were associated with Idere and Kano accessions, respectively, although the crude protein was highest in these accessions. However,

Table 5. The main effect of accession, manure and watering interval on the leaf proximate composition of *Moringa oleifera*.

Accessions	Ash (%)	Carbohydrate (%)	Crude protein (%)	Oil (%)	Crude fiber (%)	Moisture (%)
Awo-anekpa	6.1	41.1	20.3	1.8	9.4	20.7
Idere	7.6	32.5	25.6	2.3	11.9	19.9
Kano	5.5	35.9	26.9	3.0	9.8	19.1
$LSD_{(0.05)}$	0.7	3.1	2.6	0.5	1.9	1.1
Manure (t/ha)						
0	6.4	36.9	25.1	2.7	10.6	19.2
10	6.1	36.7	23.1	2.5	10.1	20.3
20	6.7	35.9	24.6	2.0	10.4	20.2
$LSD_{(0.05)}$	ns	ns	ns	0.5	ns	ns
Watering intervals (days)						
3	6.8	35.2	24.8	2.5	11.2	19.3
4	6.2	37.3	23.1	2.5	9.8	20.0
5	6.1	37.0	24.9	2.2	10.1	20.4
F-$LSD_{(0.05)}$	ns	ns	ns	ns	ns	ns

ns = non-significant

Table 6. The interaction effect of manure rate and watering interval on the leaf proximate composition of *Moringa oleifera*.

Manure (t/ha)	Watering intervals (days)	Ash (%)	Carbohydrate (%)	Crude protein (%)	Oil (%)	Crude fiber (%)	Moisture (%)
	3	6.4	34.9	26.0	2.8	13.0	18.5
0	4	7.1	36.0	24.1	3.0	8.8	19.0
	5	5.6	39.8	25.2	2.4	9.9	20.1
	3	6.4	35.2	24.6	2.3	10.2	19.8
10	4	5.8	38.4	21.4	2.7	9.9	20.4
	5	6.0	36.6	23.4	2.3	10.3	20.8
	3	7.7	35.4	23.9	2.4	10.4	19.6
20	4	5.7	37.6	24.0	1.7	10.6	20.6
	5	6.7	34.5	26.0	1.8	10.2	20.5
$LSD_{0.05}$		1.3	ns	ns	ns	ns	ns

ns = non-significant

oil content was highest with Kano accession. Manure rate only influenced the oil content of the leaf (Table 5). The oil content decreased with the application of pig manure. While, the highest leaf oil content (2.7%) was obtained with no manure application, the lowest (2.0%) was recorded when 20 t/ha manure was applied. The ash, carbohydrate, crude protein, crude fibre, oil and moisture contents of the leaf did not differ with watering interval (Table 5). The interaction of manure rate and watering interval only influenced the leaf ash content (Table 6). The application of 20 t/ha manure and 3 days watering interval resulted in the accumulation of 7.7% leaf ash content, compared to values of 5.6 to 7.1% obtained in other combinations. The least ash content of 5.6% was recorded in the control plants (which received 0 t/ha of manure) with 5 days watering interval. The interaction effect of accession, manure and watering interval on the leaf proximate composition of *M. oleifera* showed significant ($p < 0.05$) variations with respect to carbohydrate, crude protein and oil content (Table 7). However, there was no consistent trend in the variation. The range of the carbohydrate, crude protein and oil content were 25.4 to 46.1, 14.5 to 32.8, and 1.3 to 5.1%, respectively. For carbohydrate, the highest value of

Table 7. The interaction effect of accession, manure rate and watering interval on the leaf proximate composition of *Moringa oleifera*.

Accession	Manure (t/ha)	Watering intervals(days)	Ash (%)	Carbohydrate (%)	Crude protein (%)	Oil (%)	Crude fiber (%)	Moisture (%)
	0	3	5.9	40.3	20.1	1.3	12.4	18.3
		4	7.5	38.9	22.7	1.6	9.2	19.8
		5	5.4	41.4	20.3	2.0	10.3	20.5
	10	3	5.5	45.1	14.5	2.1	9.8	22.8
Awo-anekpa		4	5.6	37.7	22.5	2.2	9.6	22.5
		5	5.8	38.2	21.8	2.2	8.9	22.0
	20	3	7.6	39.6	20.8	1.9	8.5	20.5
		4	5.5	43.0	20.9	2.1	9.0	19.4
		5	6.3	45.3	19.7	1.3	7.0	20.4
	0	3	8.3	33.2	25.2	4.0	16.0	18.9
		4	8.4	33.1	28.0	2.3	9.8	18.2
		5	6.4	31.7	28.2	1.7	11.2	20.5
	10	3	8.0	25.4	30.0	2.0	11.6	18.9
Idere		4	6.9	40.4	19.2	2.8	10.1	19.5
		5	6.8	34.3	22.3	2.3	13.2	20.8
	20	3	10.0	27.0	28.5	2.6	12.9	18.7
		4	5.9	35.5	21.7	1.5	13.2	22.1
		5	7.5	32.1	27.5	1.5	9.5	21.7
	0	3	5.0	31.1	32.8	3.0	10.5	18.3
		4	5.6	35.9	21.6	5.1	7.5	18.9
		5	5.1	46.1	27.3	3.6	8.3	19.3
	10	3	5.7	35.2	29.2	3.0	9.4	17.6
Kano		4	5.1	37.2	22.5	3.3	9.9	19.3
		5	5.5	37.2	26.3	2.4	8.8	19.6
	20	3	5.7	39.6	22.4	2.6	9.9	19.6
		4	5.9	34.5	29.4	1.5	9.8	20.2
		5	6.5	26.3	30.9	2.7	14.2	19.3
$LSD_{0.05}$			ns	9.5	8.0	1.5	ns	ns

ns = Non-significant.

46.1% was obtained in the Kano accession with 0 t/ha of manure and 5 days watering interval. Similarly, carbohydrate was seemingly high in Awo-anekpa accession particularly with the application of 20 t/ha of manure. The Kano accession recorded the highest values for protein and oil contents irrespective of the manure and watering treatments.

DISCUSSION

Most of the parameters for seedling emergence, early growth and leaf proximate composition of *M. oleifera* distinctively differed with the accessions. Awo-anekpa accession had the highest emergence rate and number of emerged seedlings, indicating that it germinated faster than Idere and Kano accessions. However, the proximate qualities showed that no accession had a preponderance of the different proximate qualities rather each accession had some distinct proximate qualities it accumulated the most. For instance, Idere accession accumulated the highest ash and crude fibre, which implied that Idere contained more mineral elements than the other two accessions. Awo-anekpa had the highest carbohydrate

and moisture contents while the highest crude protein and oil content were recorded in Kano accession. These observations could probably be due to variations in the genetic potentials of the accessions and/or the inherent variability across the collection environments. The accessions were collected from three locations belonging to different ecological zones. Ugwuoke et al. (2001) earlier reported that different agro-ecologies may differ in climatic and edaphic factors. The authors averred" that varying weather and soil conditions might result to varying nutrient concentrations in the different plant parts including the seeds.". Therefore, the variability observed in the accessions may not be unconnected with the ecological zones from where they were collected. Variability observed in the performances of the accessions also suggests that source of seed or seed collection centre could influence the quality of the seedlings thereof. The chemical compositions of the different accessions also showed that the crude fat contents were low, ranging from 1 to 4%, and this may be advantageous for obese sufferers (Lintas, 1992). Low fat foods are known to reduce cholesterol level (Wardlaw and Hampl, 2006). The present study also revealed that the *M. oleifera* accessions had moderate crude fibre content (the indigestible carbohydrate component that aids digestion and reduces diabetes and high levels of blood cholesterol). The range of 9.4 to11.9% obtained in this study is in agreement with the crude fibre value reported by Olugbemi et al. (2010). *M. oleifera* leaves, in the study, also contained high amount of crude protein ranging from 20.3 to 26.9%, which also falls within the crude protein value of 24.44% reported by Olugbemi et al. (2010). However, Mutayaba et al. (2011) reported much higher value of 30.65%. Crude protein is known to play a vital role in body building; and the protein content of *Moringa* leaves is adequate in controlling malnutrition in children and enough to support breast feeding mothers during their lactating months (Duke, 1983).

The application of manure increased plant height, number of leaves and stem girth of the plants. This observation agrees with earlier reports of Baiyeri and Tenkouano (2007), Ndukwe et al. (2011) and Aba et al. (2011) that animal manure is a valuable source of crop nutrients and organic matter, which can improve the soil biophysical conditions making the soil more productive and sustainable for plant growth. Chukwuka and Omotayo (2009) specifically noted that application of organic fertilizers significantly improves the soil chemical properties and nutrient uptake in plants, thereby enhancing plant growth. In the present study, the growth parameters were significantly highest with 20 t/ha of pig manure. Increased application of the organic fertilizer was found to favour vegetative growth in the plants, and this corroborates with the findings of Ewulo et al. (2008). For the leaf proximate composition, manure application particularly influenced the oil content. The oil content of the Moringa leaves obtained with 20 t/ha manure (2.0%) was significantly smaller than the values obtained with 10 t/ha (2.5%) and 0 t/ha (2.7%) manure rates. Irrigation water application at different intervals showed no significant effect on the plant height and number of leaves but only influenced the stem girth. The non-significant effect of watering on most growth traits could probably be due to the short water stress intervals applied in this study, vis-à-vis a blanket application of dry grass mulch, which must have conserved sufficient moisture for the test plants. Takano (2004) had earlier reported that *M. oleifera* could withstand severe drought once it had fully established. Evidence from the present study indicate that watering at 3 to 5 days interval could sustain adequate growth and quality of *M. oleifera* as potted plants.

Interactions between accessions, manure and watering (second order interaction) significantly influenced carbohydrate, crude protein, and oil contents. Heywood (2002) reported that variations in essential chemical compositions can occur as a result of differing soil conditions, seasonal fluctuations and other environmental factors. The slight inconsistency observed in the second order interaction may probably be due to the short watering intervals applied in the study. As such, wider watering intervals re-evaluated with these accessions under varied manure rates may probably elicit a clearer response. We conclude from the available data that *M. oleifera* plants could be grown as potherbs with organic soil amendment (20 t/ha of pig manure) and irrigation intervals of 3 to 5 days.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Provenance variation in growth and genetic potential of *Aquilaria malaccensis* under nursery condition

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Aquilaria malaccensis **Lam. is commonly known as Agarwood which is distributed in the Indo-malesian genus Aquilaria of family Thymelaeaceae known to produce resin-impregnated heartwood that is fragrant and highly valuable. Agarwood is reputed to be the most expensive wood in the world. Twenty two open pollinated families in *A. malaccensis* were selected and evaluated for growth attributes and genetic divergence. The experiment was conducted at Forest College and Research Institute, Tamil Nadu Agricultural University; Mettupalayam situated at 11° 19' N longitude, 76° 56' E latitude at 300 MSL during January - December 2010. The study indicates significant differences among the selected families for various growth attributes. Among the twenty two progenies evaluated, three progenies viz., NHJA, KHOW-1 and CHEK-1 exhibited consistent superiority over growth periods for shoot length, collar diameter and number of branches. Genetic divergence studies resulted in grouping of the selected families into six clusters which indicated the existence of adequate genetic divergence. Among the clusters, cluster VI was the largest with 9 progenies while the maximum intra clusters distance was recorded in cluster V. The intra and inter cluster distance revealed that maximum inter cluster distance was recorded between cluster IV and V which indicated the presence of wide genetic distance between *A. malaccensis* progenies. Among the various growth attributes, number of branches contributed maximum towards genetic divergence followed by shoot length. These two characters could act as a reliable indicator for future improvement programme in this economically important species. Genetic analysis of the progenies indicated adequate variability in the population. The phenotypic co-efficient of variation (PCV) and genotypic co-efficient of variation (GCV) estimates exhibited superiority of number of branches followed by shoot length and collar diameter. In case of shoot length, collar diameter and number of branches exhibited moderate heritability. The genetic advance was high for shoot length followed by number of branches which indicated the reliability of these two parameters for inclusion in future improvement programme.**

Key words: *Aquilaria malaccensis,* provenances, variability, heritability, genetic divergence, intra and inter cluster distance.

INTRODUCTION

Aquilaria malaccensis Lam. is one of the 15 tree species in the Indomalesian genus Aquilaria of family Thymelaeaceae and eight are known to produce resin-impregnated heartwood that is fragrant and highly valuable (Ng et al., 1997). There are many names for this resinous wood, including agar, agarwood, aloe(s) wood, eaglewood, gaharu and kalamabak. This wood is in high demand for medicine, incense and perfume across Asia

and the Middle East. The tree grows in natural forests at an altitude of a few meters above sea level to about 1000 m, and it grows best around 500 m in locations with average daily temperatures of 20 to 22°C (Afifi, 1995; Keller and Sidiyasa, 1994; Wiriadinata, 1995). *Aquilaria* sp. has adapted to live in various habitats, including those that are rocky, sandy or calcareous, well-drained slopes and ridges and land near swamps. It is a large evergreen tree, growing over 15 to 40 m tall and 0.6 to 2.5 m in diameter, and has white flowers (Chakrabarty et al., 1994; Sumadiwangsa, 1997). The 2002 IUCN Red List classifies this species as vulnerable. *A. malaccensis* occurs mostly in the foothills of the North-eastern region (Assam, Meghalaya, Nagaland, Mizoram, Manipur, Arunachal Pradesh and Tripura) and West Bengal up to an altitude of 1000 m. In Assam and Meghalaya, it occurs sporadically in the district of Sibsagar, Sadiya, Nowgong, Darrang, Goalpara, Garo Hills and Cachar (Atal and Kapoor, 1982). A report by Chakrabarty et al. (1994) documenting India's trade in agarwood concluded that *A. malaccensis* is highly threatened in that country due to exploitation of the species for commercial purposes. *A. malaccensis* is threatened in its natural habitat because of overexploitation.

Demand for agarwood has resulted in the unsustainable harvesting of the species, leading to local extinctions. Wild agarwood was heavily extracted from Arunachal Pradesh between the late 1950s and the early 1980s, virtually exhausting the natural stock. Wild *A. malaccensis* is now considered almost extinct in Assam. Surveys undertaken by the Regional CITES Management Authority in Tripura indicate that the natural stock is almost exhausted in that State as well. In Mizoram, the lack of agarwood plantations in Mizoram and Meghalaya has resulted in much illegal harvesting from natural forests. *A. malaccensis* in Nagaland and Manipur is so depleted that a large proportion of the raw agarwood used by processing units in these two States is sourced from neighbouring countries. Because of its vast natural distribution and the diversity of ecological conditions where the species occurs, *A. malaccensis* would be expected to have considerable genetic variation (Shivkumar and Banerjee, 1986). Knowledge of variability within a species is a prerequisite for developing effective tree improvement / breeding strategies (Vakshasya et al., 1992). The significance of genetic variation studies and provenance testing in forest tree improvement is well realized.

Success in the establishment and productivity of forest tree plantations is governed largely by the species used and the source of seed within species (Larsen, 1954; Lacaze, 1978). No matter how sophisticated the breeding techniques, the largest, cheapest and fastest gains in most forest tree improvement programs will accrue if use of suitable species and seed sources within species is assured (Zobel and Talbert, 1984). Provenance research is therefore of paramount importance. Provenance is defined as a subdivision of species consisting of genetically similar individuals, related by common descent and occupying a particular territory to which it has become adapted through natural selection.

Therefore, present investigation has been carried out to estimate genetic variation present in *A. malaccensis* populations and survival percentage of species at Forest college and Research institute, Mettupalayam, Tamil Nadu.

MATERIALS AND METHODS

Selection of superior genetic resource

The survey has been conducted in predominant *A. malaccansis* growing areas of India and twenty two different provenances from North-Eastern states of Assam (6), Tripura (10) and Nagaland (6) were selected. Based on morphological characters such as diameter, height, number of branches and clear bole height of superior Agar wood, genetic resources were selected and measurement were recorded as given in the Table 1.

Geographical location of the population

Six provenances from Assam (MDLY, UDLY-1, UDLY-2, NHJA, NHSU, and HAKH), Ten Provenances from the state of Tripura (KHOW-1, KHOW-2, AMBS, CENT-1, CENT-2, KUMA-R, KUMA, FUKO, KUMA-RO, ROWA) and six provenances (DI-FC, DI-TY, DIPU, CHEK-1, CHEK-2, CHEK-3) from Nagaland were used for this study. Geographic locations, altitude, locations of twenty two different provenances were seeds are collected for study are cited in Table 2.

Experimental site description

The experiment was conducted at Forest College and Research Institute, Tamil Nadu Agricultural University, Mettupalayam situated at 11^{o} 19'N longitude and 76 ^{o}56'E latitude at 300 msL during January to July 2013. The experimental site receives an annual rainfall of 800 mm/annum with the maximum and minimum temperature of 33.8 and 21.2°C, respectively. The soil is predominantly red lateritic with a pH of 7.1.

Nursery technique and seedling establishment of each provenance

Pretreated seeds were directly sown in polythene bags (20 × 40 cm size) containing potting mixture of sand, soil and farmyard manure in the ratio of 2:1:1 and watered regularly as and when required.

Experimental design and treatment

The nursery experimental trail was laid out using a Completely

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Table 1. Morphological characters of superior genetic resources of Agar wood.

S/N	Provenance name	Location	GBH (cm)	Height (m)	Clear bole Height (m)	Number of Branches
1	MDLY	Modhertally	52.20	7.50	4.50	16
2	UDLI-1	Udali-1	68.00	16.5	4.00	20
3	UDLI-2	Udali-2	70.00	14.0	5.00	20
4	NHJA	Nahaurani-Jangoan village	89.00	19.5	9.00	21
5	NHSU	Nahaurani- Sumoni	81.00	19.0	5.00	17
6	HAKH	Hatiekhowa village	79.00	17.5	6.50	18
7	KHOW-1	Khowai-1	103.0	18.5	7.00	22
8	KHOW-2	Khowai-2	100.0	19.0	7.50	20
9	AMBS	Ambassa	390.0	27.0	7.00	52
10	CENT-1	Central nursery-1	110.0	18.0	12.0	10
11	CENT-2	Central nursery-2	127.0	22.0	11.0	17
12	KUMA-R	Kumargath-RFO house	147.0	25.0	10.0	18
13	KUMA	Kumargath	88.00	18.0	7.00	22
14	FUKO	Fukirkohi	87.00	16.0	2.00	18
15	KUMA-RO	Kumargath-range office	230.0	28.0	3.80	96
16	ROWA	Rowa	81.00	13.0	3.00	15
17	DI-FC	Dimapur-Forest colony	72.00	16.5	4.00	16
18	DI-TY	Dimapur- Tykho village	58.00	12.0	6.00	18
19	DIPU	Diphupur	72.00	17.5	7.50	16
20	CHEK-1	Chekieye village-1	87.00	18.0	6.50	24
21	CHEK-2	Chekiye village-2	75.00	17.0	7.00	17
22	CHEK-3	Chekiye village-2	83.00	16.5	7.00	18

Table 2. Details of location, latitude, longitude, elevation (m) of superior genetic resources of Agar wood provenance.

S/N	Provenance name	Location	State	Latitude	Longitude	Elevation (m)
1	MDLY	Modhertally	Assam	$26^0$08.100	$92^0$49.771	123
2	UDLI-1	Udali-1	Assam	$25^0$53.304	$93^0$00.604	89
3	UDLI-2	Udali-2	Assam	$25^0$53.303	$93^0$00.600	90
4	NHJA	Nahaurani-Jangoan village	Assam	$26^0$38.856	$94^0$03.318	80
5	NHSU	Nahaurani- Sumoni	Assam	$26^0$38844	$94^0$03.348	78
6	HAKH	Hatiekhowa village	Assam	$26^0$36.476	$94^0$01.826	82
7	KHOW-1	Khowai-1	Tripura	$24^0$04.186	$91^0$36.868	40
8	KHOW-2	Khowai-2	Tripura	$24^0$06.170	$91^0$36.840	47
9	AMBS	Ambassa	Tripura	$23^0$55.138	$91^0$50.522	74
10	CENT-1	Central nursery-1	Tripura	$23^0$54.891	$91^0$53.144	115
11	CENT-2	Central nursery-2	Tripura	$23^0$54.927	$91^0$53.175	126
12	KUMA-R	Kumargath-RFO house	Tripura	$24^0$10.501	$92^0$01.922	38
13	KUMA	Kumargath	Tripura	$22^0$09.695	$92^0$02.661	39
14	FUKO	Fukirkohi	Tripura	$24^0$10.700	$92^0$01.288	32
15	KUMA-RO	Kumargath-range office	Tripura	$24^0$10717	$92^0$01.923	61
16	ROWA	Rowa	Tripura	$24^0$22.084	$98^0$49.328	97
17	DI-FC	Dimapur-Forest colony	Nagaland	$25^0$54.733	$93^0$42.825	152
18	DI-TY	Dimapur- Tykho village	Nagaland	$25^0$53.189	$93^0$43.271	158
19	DIPU	Diphupur	Nagaland	$25^0$51.294	$93^0$45.493	160
20	CHEK-1	Chekieye village-1	Nagaland	$25^0$51.856	$93^0$45.049	162
21	CHEK-2	Chekiye village-2	Nagaland	$25^0$51.863	$93^0$45.479	164
22	CHEK-3	Chekiye village-2	Nagaland	$25^0$51.871	$93^0$45.488	165

Randomized Block Design with 22 provenances for 3 replications. Observation with respect to survival percentage, shoot, collar diameter and numbers of branches were taken at every one month interval till the end of experiment (6 months) in order to, assess the suitable provenances and their survival percentage before planting in main field.

Biometrical observation

Mean performance of progenies

Survival percentage: Survival of seedlings was calculated and expressed as percentage.

$$\text{Survival percentage (\%)} = \frac{\text{No. of survival seedling}}{\text{No. of seedlings planted in nursery}} \text{ X } 100$$

Measurements: Shoot length, Collar diameter of individual seedlings was measured and numbers of branches was counted at an interval of one month and observation was recorded.

Variability studies: These parameters were estimated as per the method described by Johnson et al. (1955).

Phenotypic co-efficient of variability: Phenotypic Co-efficient of Variation (PCV) was arrived by using the formula as described by Burton (1952).

$$\text{PCV (\%)} = \frac{(\text{Phenotypic Variance})^{1/2}}{\text{General Mean}} \times 100$$

Genotypic co-efficient of variability: Genotypic Co-efficient of Variation (GCV) was arrived by using the Burton's (1952) formula.

$$\text{GCV(\%)} = \frac{(\text{Genotypic Variance})^{1/2}}{\text{General Mean}} \times 100$$

Heritability (h^2): Broad sense heritability (h^2) was calculated according to Lush (1940)

$$h^2 = (\sigma^2 g / \sigma^2 p)$$

Heritability percentage = h^2 x 100

Genetic advance: Genetic advance was worked out after Johnson et al. (1955a).

$$\text{Genetic Advance (GA)} = [(\text{Genotypic Variance} / \text{Phenotypic Variance})^{1/2} \times K$$

Where, K = 2.06, a selection differential at 5% selection intensity

$$\text{GA(\%)} = \frac{\text{GA}}{\text{Grand Mean}} \times 100$$

Data analysis

Biometric data for shoot length, collar diameter and number of branches were subjected to analysis of variance (Panse and Sukhatme,1978) and genetic divergence of the open pollinated families was studied following Mahalanobis D^2 (Mahalanobis, 1928) statistics. Grouping of the superior open pollinated families into various clusters was made by Tocher's method (Rao, 1952). On completion of clustering, the *intra* and *inter* cluster relationships were studied and the mutual relationship between clusters and their distances were represented. The average *intra* cluster distance was measured using the formula. $D^2 = D_1^2 / n$ where D^2 was the sum of the distances between all possible combinations of the open pollinated families included in a cluster whereas the average *inter* cluster divergence was arrived at by taking into consideration all the component D^2 values possible among the numbers of the two clusters. The genetic distance D between the clusters was obtained from the square root of the average D^2 values. Estimation of genetic parameters *viz*., variability, PCV and GCV were computed (Burton, 1952). Heritability and genetic advance were computed (Lush, 1940; Johnson et al., 1955).

RESULTS AND DISCUSSION

Mean performance of *A. malacansis* genotypes

Success in the establishment and the productivity of forestry plantation is governed largely by the species used and the source of seed within species (Larson, 1954; Lacaze, 1978). No matter how sophisticated the breeding techniques, the largest, cheapest and fastest gains in most forestry improvement programmes will accrue if use of suitable species and seed sources within species is assured (Zobel and Talbert, 1984). Seeds were much influenced by their place of origin (Heydecker, 1972) especially due to environmental variation in latitude, altitude, rainfall, temperature, moisture, soil and the external factors (Holzer, 1965). The seed source variations were reported on many tree species (Shivakumar and Banerjee, 1986; Murthy, 1989; Masilamani and Dharmalingam, 1999) and were dictated by environmental and edaphic factors. This might also be due to altitudinal variation (Barnett and Farmer, 1978) or region of collection (Bonner, 1984). Significant differences among provenances were detected for survival percentage. The survival rate showed a decreasing trend with decreasing latitude of provenance The survival percentage of provenance ranged from 21 (AMBS) to 42% (NHJA and CHEK-1). The highest mean survival (42%) was recorded in NHJA and CHEK-1 followed by UDLI-2 AND KHOW-1 (41.66%). The provenance from AMBS-1 (21%) followed by UDLI-1, HAKH, DI-FC and CHEK-2 (23.66%) had lowest survival percentage (Table 3). In the present investigation, significant variation was observed for all the attributes viz., shoot length, collar diameter and number of branches at nursery level for 22 progenies of *A. malacancis*. Among the progenies, the superiority of three progenies viz., NHJA, KHOW-1 and CHEK-1 was evident for most of the growth characteristic investigated (Table 4). The shoot length of Agarwood provenances were observed to increase in shoot length with increase in number of days of observation.

Table 3. Survival percentage of 22 provenance of Agar wood.

Provenance number	Provenance name	Survival percentage (%)
1	MDLY	34.33
2	UDLI-1	23.66
3	UDLI-2	41.66
4	NHJA	42.00
5	NHSU	33.00
6	HAKH	23.66
7	KHOW-1	42.00
8	KHOW-2	35.33
9	AMBS	21.00
10	CENT-1	24.33
11	CENT-2	33.33
12	KUMA-R	33.66
13	KUMA	20.66
14	FUKO	25.33
15	KUMA-RO	31.33
16	ROWA	32.00
17	DI-FC	23.33
18	DI-TY	27.33
19	DIPU	25.00
20	CHEK-1	41.66
21	CHEK-2	23.66
22	CHEK-3	34.33
	Mean	30.98
	SE.d	1.483
	CD (0.05)	2.99 0

Table 4. Morphological characters of superior genetic resources of Agarwood.

Provenance number	Provenance name	Shoot length (cm)		Collar diameter (mm)		Number of branches	
		120 DAP	180 DAP	120 DAP	180 DAP	120 DAP	180 DAP
1	MDLY	18.83	40.26	5.533	11.12	1.000	3.000
2	UDLI-1	21.83	37.93	5.816	10.70	1.000	2.000
3	UDLI-2	19.73	41.16	5.666	11.10	1.333	2.000
4	NHJA	23.26	48.16	4.383	11.16	1.000	2.000
5	NHSU	19.70	32.66	5.950	11.06	1.000	4.000
6	HAKH	18.70	39.33	5.350	11.05	0.000	1.000
7	KHOW-1	24.50	45.00	5.833	11.12	0.666	2.000
8	KHOW-2	18.56	27.50	4.190	10.90	1.000	2.666
9	AMBS	18.56	27.50	4.190	11.01	1.000	2.000
10	CENT-1	18.40	36.93	4.926	11.02	0.666	3.000
11	CENT-2	17.10	32.00	3.983	10.93	1.333	3.000
12	KUMA-R	18.53	37.26	4.136	11.18	1.000	2.000
13	KUMA	16.40	28.40	5.566	10.80	0.666	3.000
14	FUKO	18.50	30.83	5.466	11.01	0.666	4.000
15	KUMA-RO	16.56	24.00	6.133	11.03	0.666	4.000
16	ROWA	17.90	30.16	5.433	11.09	1.333	3.000
17	DI-FC	18.86	31.00	5.333	11.14	1.000	2.000
18	DI-TY	20.43	33.56	5.216	11.10	0.666	4.000
19	DIPU	15.73	25.00	5.600	11.14	0.666	3.000

Table 4. Contd

20	CHEK-1	18.46	36.66	5.766	11.16	1.000	3.000
21	CHEK-2	17.13	39.23	5.766	11.26	0.666	3.000
22	CHEK-3	14.53	25.50	4.766	11.14	1.333	3.000
	Mean	18.68	34.47	5.163	11.05	0.924	3.030
	SE.d	1.259	6.117	0.606	0.158	0.471	0.864
	CD (0.05)	2.537	12.32	1.222	0.318	0.950	1.742

Table 5. Clustering pattern in *Aquilaria malaccansis* for morphometric attributes.

Cluster number	Number of family	Members
I	3	MDLY, NHJA, KUMA-R
II	2	NHSU, FUKO
III	2	UDLI-2 , KHOW-1
IV	2	DIPU , CHEK-3
V	4	UDLI-1, HAKH, KHOW-2, CENT-1
VI	9	AMBS, CENT-2, KUMA, KUMA-RO, ROWA, DI-FC, DI-TY, CHEK-1, CHEK-2

Provenances only differed significantly in mean shoot length. At 120 DAP, the length of shoot varied and ranged from KHOW-1 (24.50 cm) to KUMA (16.40 cm). At 180 DAP, the provenance exhibited significant variation in shoot length ranged between NHJA (48.16 cm) and FUKO (30.83 cm). NHJA (48.16 cm) and KHOW-1 (47 cm) were recorded significantly higher shoot length compared to general mean (Table 4). All other provenances were on par with general mean for this parameter.

At 120 DAP, the collar diameter ranged between NHJA (6.133 cm) and CENT-1 (3.983 cm). Other than NHJA provenance, all provenances were on par with general mean for the collar diameter. At 180 DAP, two provenance viz., NHJA (48.16 cm) and CHEK-1 (11.36 cm) were recorded significantly higher value and UDLY-1 (10.70 cm) had significantly lower value compared to general mean (Table 4). The provenance HAKH (0.000) had not produced any branches and all other provenances were on par with general mean for numbers of branches at 120 DAP. At 180 DAP, number of branches varied between NHJA, KHOW-1, CHEK-1, DI-TY (4.000) and HAKH (1.000). HAKH (1.000) were recorded significantly lower value compared general mean (Table 4). Similarly in teak variations in several growth characters, stem and morphological characters were evident due to provenance (Rawat et al., 1998). A plethora of workers reported on the existence of variations in morphometric traits of various tree species like *Dalbergia sissoo* (Tewari et al., 1996), *Eucalyptus tereticornis* (Otegbeye, 1990), *Santalum album* (Bagchi and Sindu Veerendra, 1991), *Tecomella undulata* (Jindal et al., 1991) *Lagerstroemia* spp. (Jamaludheen et al., 1995) and *Terminalia arjuna* (Srivastava et al., 1993).

Cluster composition

Clustering methods have the goal of separating a pool of observations in many subgroups to obtain homogeneity within and between the formed subgroups. D^2 statistics is an important tool in plant breeding for estimating genetic divergence (Aslam Mohd et al., 2011). D^2 statistics is an important tool in plant breeding for estimating genetic divergence. The application of D^2 clustering technique in *A. malacancis* genetic resources resolved the twenty two genotypes into six clusters. Among the six clusters, the clusters VI and V were the biggest with 9 and 4 members, respectively. The Cluster I contains 3 members and remaining cluster constitutes two progeny each (Table 5). In *Tectona grandis* using D^2 clustering technique 80 batches of teak had been grouped into eight clusters, of which group A formed the largest cluster containing 46 batches (Bagchi, 2000). And also, *Melia dubia* has been grouped into six clusters in that cluster I formed biggest group (Kumar et al., 2013). In the present investigation it was observed that the families from different locations got clubbed together to form a single major cluster as evident in cluster I and therefore the pattern of divergence was not dependent upon the geographic locations. Inclusion of geographically divergent provenances of teak in the same cluster may be attributed to the fact that the factors other than geographic distribution might be responsible for their genetic similarity (Subramanian et al., 1994).

Intra and inter cluster average distance

The *intra* and *inter* cluster analysis indicated that this may be due to introduction and demonstration during past

Table 6. Inter and intra cluster distances for morphometric attributes.

Cluster	1	2	3	4	5	6
I	0.48437	3.01125	0.43116	3.70187	3.29812	3.03829
II		0.12625	4.17527	1.97394	5.21623	1.69394
III			0.19793	4.90531	2.65486	3.87403
IV				0.22385	7.96942	2.2769
V					3.47435	5.64495
VI						2.86515

Table 7. Inter (diagonal) and intra cluster D2 values for morphometric attributes.

Cluster	1	2	3	4	5	6
I	0.69597	1.73529	0.65663	1.92402	1.81607	1.74307
II		0.35532	2.04335	1.40497	2.28391	1.30151
III			0.44489	2.21479	1.62937	1.96825
IV				0.47313	2.82302	1.50894
V					1.86396	2.37591
VI						1.69268

Table 8. Percentage contributions of morphometric traits to genetic divergence at -180 DAP.

Characters	Number of first rank	% contribution
Shoot length	85	36.79
Collar diameter	15	6.493
Number of branches	131	56.71
Total	231	100

years as evidenced in *Bombax ceiba* (Chaturvedi and Pandey, 2001). The maximum *intra* cluster distance was shown by the cluster II (1.803). The average intra and inter cluster D^2 and D values among the six clusters are presented in Table 6 and 7. The maximum intra cluster distance was shown by the cluster V (3.474) followed by cluster VI (1.692). From the inter cluster distance, it is inferred that the cluster I and III (0.484) were the closest while the maximum inter cluster distance was recorded between Cluster IV and V (7.969) which indicated the presence of wider genetic distance between *A. malaccansis* families. Such inter and intra cluster distance among *Pinus gerardiana* (Anilkant et al., 2006) and *M. dubia* reported which lend support to the current findings (Kumar et al., 2013).

Contribution of traits towards genetic divergence

Number of branches contributed the maximum towards genetic divergence (56.71%) followed by shoot length(36.79%) and the least by Collar diameter (6.493%) (Table 8).

Variability parameters

The assessment of genetic variability is a key to progress in tree improvement (Zobel, 1981) and is a useful tool in determining the strategies for tree improvement and breeding of any species. To understand the causes of variation, apportioning of total phenotypic variation is having more utility. The genetic variation which is heritable can be exploited for further improvement programme. In this study, number of branches registered high PCV (38.02) and GCV (15.04). Shoot length recorded moderate PCV (25.37) and GCV (14.27) followed by collar diameter PCV (1.805) and GCV (0.616) (Table 9) Higher GCV for number of branches in *E. tereticornis* and low GCV for height in the same species were earlier reported (Paramathama,1992). Similarly, low GCV and PCV for height and collar diameter were also reported in *Bambusa pallida* (Singh and Beniwal, 1993). The exhibition of low to moderate PCV and GCV for

Table 9. Genetic estimates for growth attributes at 180 DAP.

Traits	GCV	PCV	ECV	Heritability	Genetic advance (%)
Shoot length	14.27	25.37	20.98	0.316	16.54
Collar diameter	0.616	1.805	1.697	0.116	0.433
Number of branches	15.04	38.02	34.92	0.156	12.25

collar diameter and shoot length in the present study is in conformity with the above assertions. The genotypic and phenotypic coefficient of variation for shoot length, collar diameter and number of branches recorded in the current study provided evidences for existence of adequate genotypic variations (Kumar et al., 2010) and thus extend the scope for exploitation of genetic variability for further improvement in this multiple utility species. The relative values of PCV and GCV give an idea about the magnitude of variability present in a genetic population. In the current study, the estimates of GCV were less than PCV for many traits indicating the role of environment in the expression of the traits. The variability parameter estimates in the study are in close approximation with the findings of genetic parameters in *Azadirachta indica* (Dhillon et al., 2003), *Pongamia pinnata* (Kumaran, 1991) and also in progenies of *Dalbergia sissoo* (Dogra et al., 2005) which lend support to the findings of current investigation.

Heritability and genetic advances

Heritability has an important place in tree improvement programme as it provides an index of relative strength of heredity versus environment. Dorman (1976) reported that heritability is very important in tree improvement programme. Heritability expresses the degree to which a character is influenced by heredity as compared to the environment (Kumar et al., 2010). Estimation of broad sense heritability for various characters showed low to moderate heritability for shoot length (0.31), number of branches (0.15) and collar diameter (0.11) (Table 9). The results are in agreement with the studies carried out in *Eucalyptus globulus* who reported low heritability for DBH during field evaluation of 8 sub races (Apiolaza et al., 2005). Similarly, low to moderate heritability was also recorded in *E. globulus* and in *Eucalyptus nitens* (Raymond, 2002) for different genetic parameters and low to moderate heritability for height and tree volume in *Eucalyptus grandis* (Osorio et al., 2001). The authors also reported that the heritability varied with changing environment and age. Though heritability in broad sense may give useful indication about the related value of selection, heritability along with associated genetic gain should be considered together for valid, reliable and useful conclusion. In the current study, the trend of genetic advance as percent of mean was maximum in Shoot length (16.54) followed by number of branches (12.25) and collar diameter (0.433) (Table 9) indicating a wide scope for genetic improvement in the species. The findings of current study are in line with those of *Heracleum candicans* (Devagiri et al., 1997). In a holistic view, the existence of adequate variability for different growth attributes coupled with low to moderate heritability indicates the possibility for identification of the best family suitable for commercial utilization.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Application of zein antimicrobial edible film incorporating *Zataria multiflora* boiss essential oil for preservation of Iranian ultrafiltered Feta cheese

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Zein based edible film was developed and incorporated with *Zataria multiflora* boiss essential oil. Mechanical and microbiological characteristics of this biofilms were measured. Increasing concentration of antimicrobial agent in film reduced stretchability, tensile strength and elongation, however increased the thickness and water vapor transmission parameters (P<0.05). Addition of *Z. multiflora* boiss essential oil, resulted in decreasing the count of viable *Salmonella enteritidis, Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus* in the produced cheese. This investigation concludes that the biofilm containing *Z. multiflora* boiss essential oil can be highly recommended for packaging of Feta cheese with the improved microbiological and sensory quality. Besides, application of natural plant essential oil has economical and health promoting benefits.

Key words: Zein, *Zataria multiflora*, cheese, pathogens, mechanical properties.

INTRODUCTION

Dairy products are nutritionally valuable parts of the human diet, the demand for which is set to grow, globally. Shelf life as an important element influencing the quality of dairy products may be shortened by microbial growth. It can be extended by applying thermal treatment during manufacture and addition of preservatives. The combination of the increasing occurrence of food-borne disease and the results of social and economic problems indicate there is a steady demand to produce new antimicrobial agents (Palmer et al., 2001). Concerns for the safety of some chemical preservatives and negative customer feedbacks over their use have encouraged increasing concentration in more natural green alternatives for the extension of product shelf-life. The antimicrobial agents might be added directly to the product formulation to reduce the growth or survival of food-borne bacteria.

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Essential oils have been known for their biological activities for many decades and they should, in principle, not be toxic to human and could replace toxic synthetic fungicides. However, the food industries have been encouraged for more application of antimicrobial agents (Arora and Kaur, 1999; Appendini and Hotchkiss, 2002; Naser-Abbas and Kadir-Halkman, 2004). The ingredients of edible films are important to reduce the growth of microorganisms. The agents of edible films show an important role to the surface of the product (Ayana and Turhan, 2009).

In the past concern of food packaging was good looking, but today new consideration are of interests including environmental impact (Joerger, 2007), efficiency of heat and mass transfer, intelligent and active packaging. Active packaging is a new conception that leads to extend the shelf life of the product or improve sensory of the product (Devlieghere et al., 2004). Incorporating anti-microbial agents in food packages (antimicrobial active packaging) could be more efficient than the direct addition of these compounds into the food. Besides, migrations of the active compound from the packaging material into the food enable not only the inhibition of food-borne bacteria present in food, but also create a remaining activity over time, during transport, storage and distribution of food (Bolgar et al., 2007).

An edible film as a primary packaging made from edible components can be applied to improve sensory, shelf life, mass transfer and mechanical properties of various food products (Krochta, 2002; Janjarasskul and Krochta, 2010) as well as health benefits (including vitamins, minerals and bioflavonoids) (Larotonda et al., 2005; Park and Zhao, 2006; Park et al., 2011). The biodegradability of edible coatings is other desirable benefits associated with their use (Krochta, 2002; Siracusa et al., 2008). Edible polymeric packaging materials can be made from polysaccharides (for chitosan, carrageenan), proteins and lipids. The edible films which are usually between 50 to 250 µm in thickness can be used as wrapping materials, stand-alone films, or can be fabricated into pouches and bags for subsequent packaging use. Edible films are distinguished from coatings by their method of manufacture and application to the food product. Films are dried preformed thin material structures that are used on or between layers of food components (Pascall and Lin, 2013).

Zein is a natural protein found in corn seeds. It is a unique and complex material, and one of the few cereal proteins extracted in a relatively pure form (Panchapakesan et al et al., 2012). Zein based films could act as barriers to oxygen, carbon dioxide, and oils, thus helping the prevention of the deterioration of food quality and extension of the shelf-life of food products. Zein is not soluble in water but soluble in 40 to 90% ethanol. The water-insoluble characteristic of zein makes it a good candidate for the development of natural biopolymeric edible films (Shukla and Cheyran, 2001).

Several studies have focused on application of herbal extracts and essential oils including antimicrobial phenolic compounds in film packaging (Shaffiee and Javadian, 1997; Seydim and Sarikus, 2007). *Z. multiflora* boiss. is a plant that belongs to the Laminaceae family and grows only in Iran, Pakistan and Afghanistan (Ali et al., 2000). Among 215 species of *Z. multiflora* (known as Thymus and ‘‘Avishan” in Persian) grown in the world, 14 species are distributed in Iranian flora (Jalas, 1982). Leaves and flowering parts of this plant are well known as medicinal plants due to their health benefits as analgesic and carminative specifications, antiseptic and carminative as well as treating colds, tonic and herbal tea (Mozzaffarian, 1998). It is used as a traditional cure for its antiseptic, analgesic, carminative specifications (Mozzaffarian, 1998) in treatment of Rheumatism, and skin disorders. Also, this plant is applicable for preservation of several food products in Iran due to strong microbial inhibition and flavoring (Hosseinzadeh et al., 2000). Its antimicrobial activity is well established against a wide variety of bacteria (Aktug and Karapinar, 1986; Karaman et al., 2001; Palmer et al., 2001; Delgado et al., 2004; Fazeli et al., 2007; Altiok et al., 2012) and fungi (Rana et al., 1997; Karman et al., 2001), and attributes to the phenolic compounds such as carvacrol and thymol (Ali et al., 2000; Bagamboula et al., 2004).

Although, there are several reports on essential oil compositions of different Thymus species, investigations on their biological activities are still scarce. Nejad Ebrahimi et al. (2008) have reported the essential oil compositions of some Iranian Thymus species. The antibacterial and antifungal activity of *Thymus revolutus* oil from Turkey (Karaman et al., 2001), *Thymus pubescens* and *Thymus serpyllum* (Rasooli and Mirmostafa, 2002) as well as antioxidant activities of the oils of *Thymus caespititius, Thymus camphorates* and *Thymus mastichina* from Portugal have been reported (Miguel et al., 2004). Antimicrobial packaging decreases the growth of some microorganisms by using the slides of the films directly on some solid foods for example, cheese (Cagri et al., 2003).

Infected cheese can cause some diseases such as Gastroenteritis and Staphylococcal food poisoning that sourced by *Escherichia coli* and *Staphylococcus aureus* (Ayana and Turhan, 2009). According to Ghasemi et al. (2012) study, there was a decline in the growth of microorganisms by increased concentration of ZEO. *Salmonella enteritidis, Listeria monocytogenes, E. coli* and *S. aureus* showed significant reductions in bacterial survival in the higher ZEO concentration.

In this investigation, *Z. multiflora* was incorporated into zein based films aiming to control the growth of *S. enteritidis, L. monocytogenes, E. coli and S. aureus.* Furthermore, this work evaluated tensile strength and gas permeability characteristics of the films. The objective of this study was to investigate the role of packaging technology in extending the shelf life of cheese and reducing the risk of the pathogens.

Table 1. Composition table of *Zataria* essential oil.

Number	Composition	*Zataria multiflora*	
		Retention time (min)	Concentration (%)
1	α-Pinene	10.3	1.4
2	Camphene	10.8	-
3	β-Pinen	11.8	-
4	β-Myrcene	12.3	0.8
5	β-Phellandrene	12.8	-
6	carene	13.2	-
7	p-Cymene	0.513	8.5
8	γ-Terpinene	14.6	6
9	α-Terpineol	19.1	-
10	Thymol	22.5	48.2
11	Carvacrol	22.8	13.8
12	Caryophyllene	25.1	-
Total			78.7

MATERIALS AND METHODS

Z. multiflora boiss. essential oil preparation

Z. multiflora boiss. was purchased from Soha4 company (Tehran, Iran). 50 g dried leaves of plant was placed into a flask and the essential oil was extracted with a clevenger-type apparatus using hydro-distillation method for 2 h until no more essential oil was obtained (Didry et al., 1993). The essential oil was collected, dried with anhydrous sodium sulphate and stored at 4°C and kept for further usage. Table 1 shows the composition of ZEO used in this study.

Gas chromatography/mass spectrometry analysis

The GC–MS analyses were performed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness) equipped with a HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization 115 (70 eV). Oven temperature initially was kept at 50°C for 5 min and raised at the rate of 3°C/min to 240°C and reached to 290°C at 15°C/min then held isothermal for 3 min. Carrier gas was Helium at flow rate of 0.8 ml/min. 1 µl of samples and external standard were injected manually in the splitless mode. The components of the oil were identified based on the comparison of their retention time and with those of literature or with those of authentic compounds available in our laboratory and confirmed by matching their mass spectra with those of a computer library of the GC– MS data system and other published mass spectra. (C8–C22) n-alkanes were used as reference points in the calculation of relative retention indices (PRI) (Adams, 1995).

Inoculum preparation

L. monocytogenes ATCC 7644, *E. coli* 0157:H7 VT negative, *S. enteritidis* and *S. aureus* ATCC 25923 were obtained from Research Department of Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran. These were maintained on nutrient agar (Oxoid CM0003) slopes at 5°C. The above mentioned microorganisms were transferred from the cultures to the slopes and incubated for 24 h in 37°C. After that the procedure for the three sub-cultures was carried out to make sure the organisms were active and vital. The organisms were subcultured three times on consecutive days in nutrient broth (Oxoid CM0001) incubated at 37°C at precise 24 h intervals, followed by streaking on nutrient agar incubated at 37°C to check purity. The inoculum is prepared using the third sub cultured, which contained ca. 10^8 cfu/ml. Decimal dilutions are made to give a concentration of ca 10^6 cfu/ml. From this a volume of 500 µl was used to inoculate the broth combinations to give a final concentration of ca 10^4 cfu/ml (Ghasemi et al., 2012).

Preparation of films

The zein films are prepared according to casting method (Dry process). Zein (5.00 g) was dissolved in 45 ml of ethanol at 78°C. Glycerol was added (1.5 ml) as a plasticizer. Different concentration of *Z. multiflora* Boiss. (0, 1, 2, 3 and 4% w/v) were added. After degassing, the films were cast on glass plates and dried overnight at 22°C (Ayana and Turhan, 2009).

Packaging of surface-contaminated cheese with antimicrobial zein films

Cheese was bought from a local market, Tehran, Iran. Four microorganisms (two gram positive and two gram negative bacteria) of *S. enteritidis, L. monocytogenes, E. coli* and *S. aureus* were used. Cheese (100 g) inoculated separately with each bacterium. The slices of cheese were wrapped with zein control edible films and zein edible films containing 1, 2, 3 and 4% (w/v) of *Z. multiflora*. The slices of cheese were put into the refrigerator at 4°C for 14 days.

Film thickness

The thickness of the films was measured by a digital micrometer (Mitu-toyo, Mitutoyo Corporation, Japan) at five different positions and the mean of the thickness was used as a film thickness.

Water vapor permeability determination

The water vapor permeability is a measure for breathability or for an

Table 2. Mechanical properties of zein films formulated with *Zataria multiflora* Boiss essential oil.

Film formulation	Thickness (mm)	Tensile strength (MP_a)	Elongation at break (%)
Control	0.100	24.27	3.64
1% ZEO	0.100	6.98	1.33
2% ZEO	0.132	4.68	1.02
3% ZEO	0.195	2.04	0.86
4% ZEO	0.242	1.24	0.50

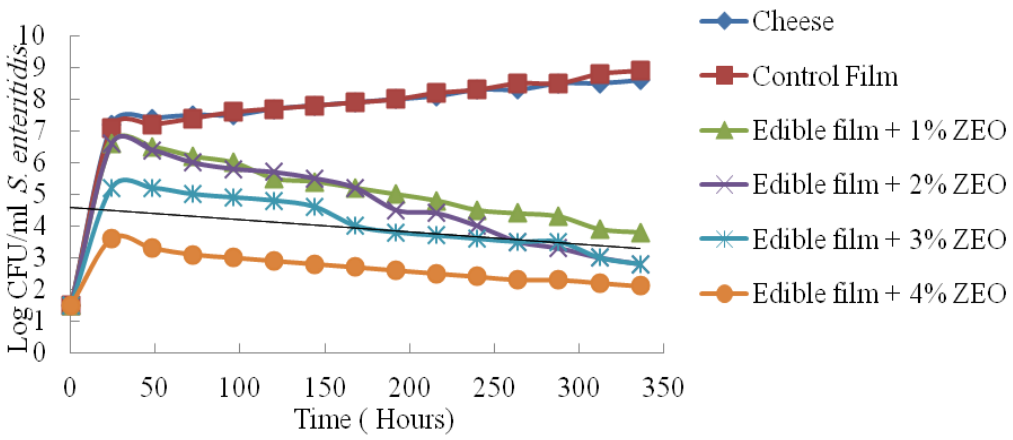

Figure 1. Inhibition of *Salmonella enteritidis* in cheese by zein edible film incorporated different concentrations of *Zataria multiflora* Boiss. essential oil.

ability of textile to transfer moisture (Hu et al., 2001). The water vapor permeability was measured according to ASTM E 96-95 (1995). A cup is filled with distilled water leaving a small gap (1.90 to 0.63 cm) of air space between the specimen and the water. The cup is then sealed to prevent vapor loss except through the test sample. An initial weight is taken of the apparatus and then periodically weighed over time until results become linear (Merck, Darmstadt, Germany). Caution must be used to assure that all weight loss is due to water vapor transmission through the specimen.

Mechanical properties of films

Mechanical characteristics were considered according to ASTM, 2001 method by using a Universal Testing Instrument Model AI-5000 (Gotech, Taiwan) fitted with a 50 N static load cell.

Statistical analysis

Each film attribute was measured in triplicate. The data were considered by SPSS software with (version 18.0, IBM, New York, NY) and Excel 2007 with a significance set at P<0.05.

RESULTS AND DISCUSSION

Mechanical properties

ZEO was added with different percentages to the Zein films to investigate the impact of oil concentration on mechanical properties of biofilm. As Table 2 shows, the thickness and mechanical properties of ZEO incorporated films will change through the incorporation of ZEO with zein films. The results show that the zein film without any combination is stronger and more stretchable than the film which was added ZEO. The films containing antimicrobial agents had thicknesses of 0.100 mm for 1% ZEO film and 0.242 mm for 4% ZEO film as evaluated with a thickness of 0.100 mm for control film. Also, the enhancement of antimicrobial agent leads to decreased tensile strength and elongation but increased films thickness (P<0.05).

Antimicrobial activity

Figures 1 to 4 show the effect of the zein films incorporated with 0, 1, 2, 3 and 4% (w/v) essential oil of *Z. multiflora* Boiss on the survival / growth of *S. enteritidis, E. coli, S. aureus* and *L. monocytogenes* over 14 days at 4°C. Results indicate a significant decrease was detected between the control films and different concentration of the essential oil on mentioned bacteria. In other words in presence of ZEO there is a sharp decrease of the bacteria in plates regarding the concentration of essential oil.

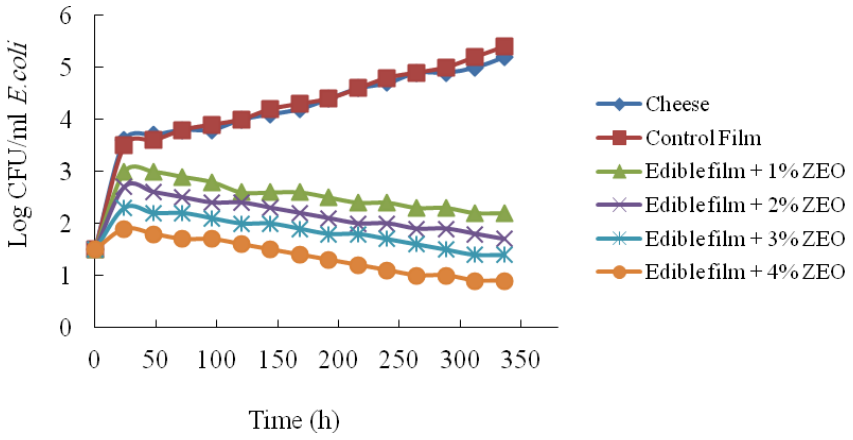

Figure 2. Inhibition of *Escherichia Coli* in cheese by zein edible film incorporated different concentrations of *Zataria multiflora* Boiss. essential oil.

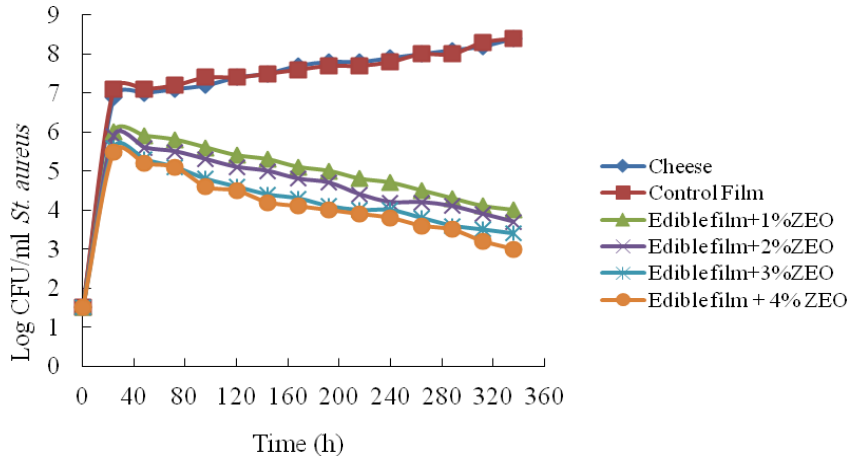

Figure 3. Inhibition of *Staphylococcus aureus* in cheese by zein edible film incorporated different concentrations of *Zataria multiflora* Boiss. essential oil.

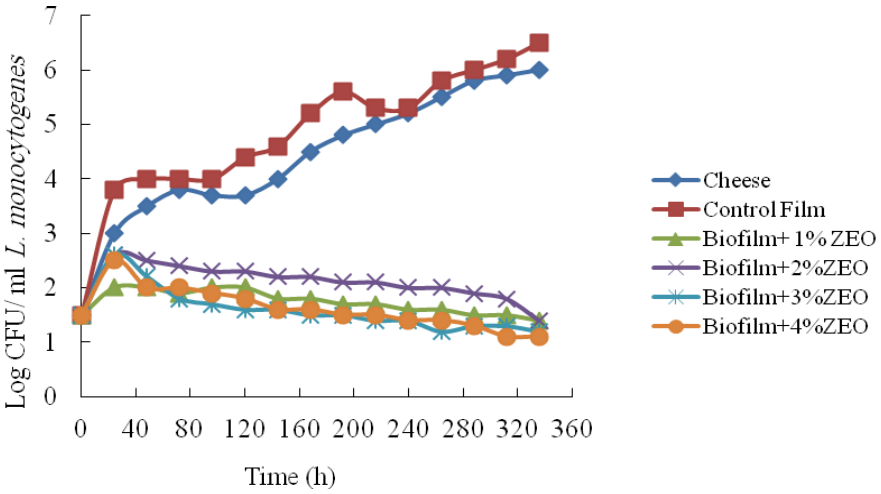

Figure 4. Inhibition of *Listeria monocytogenes* in cheese by zein edible film incorporated different concentrations of *Zataria multiflora* Boiss. essential oil.

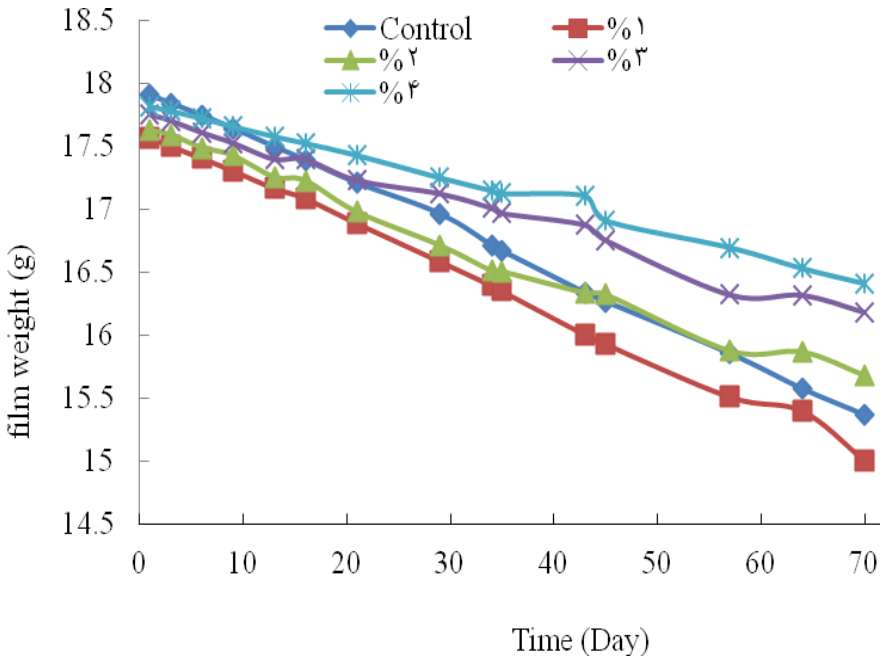

Figure 5. Water vapor transmission rate of zein films formulated with *Zataria multiflora* boiss. essential oil.

Water vapor transmission

Water vapor transmission rate (WVTR) is the steady state rate at which water vapor permeates through a film at specified conditions of temperature and relative humidity (Hu et al., 2001). The WVTR value was expressed in mg per day, of each container taken by the Equation (1):

$$(1 / N)[(WF - WI) - (CF - CI)]$$

in which *N* is the number of days expired in the test period (beginning after the initial 24 h equilibration period); (*WF* – *WI*) is the difference, in mg, between the final and initial weights of each test container; and (*CF* – *CI*) is the difference, in mg, between the average final and average initial weights of the controls, the data being calculated to two significant figures. Where the permeations measured are less than 5 mg per day, and where the controls are observed to reach equilibrium within 7 days, the individual permeations may be determined more accurately by using the 7-day test container and control container weights as *WI and CI*, respectively, in the calculation (Shimizu and Demarquette, 2000). Figure 5 shows water vapor transmission rate of zein films formulated with *Z. multiflora* Boiss essential oil (ZEO). The results show a decrease in water vapor transmission of films was observed as increasing amount of antimicrobial agent in Zein films solution. The zein films containing 4.00% (w/v) ZEO demonstrated minimum value of water vapor transmission ($P<0.05$).

DISCUSSION

Mechanical properties

It may be presumed that the films containing antimicrobial agent can affect the polymeric structure of the film and reduce the mechanical properties (Piers et al., 2008). The result was comparable with those of Ayana and Turhan (2009) who confirmed the plain films are stronger and more stretchable than the films which added some antimicrobial agents. In another study Piers et al. (2008) showed lower tensile strength and elongation to break for the films that containing antimicrobial agent than control films.

Antimicrobial activity

Results show a sharp decrease of the bacteria regarding the concentration of ZEO which is due to the presence of important phenolic components such as carvacrol and thymol. A case study by Kim et al. (1995) showed the antimicrobial effects of carvacrol on the *Salmonella* Typhimurium in culture medium and fish cube. They found that carvacrol with 3% concentration has very strong antimicrobial activity on the *S.* Typhimurium. In another study, Karaman et al. (2001) found the antimicrobial activity of the *T. revolatus* extract against some Gram positive and Gram negative bacteria. They concluded that it was because of the high quantity of the

carvacrol in *T. revolatus*. Comparable studies were reported that the protein based films with ZEO consist of some phenolics competent that reduce free radicals for rising antimicrobial activities to some products. It may be because of the effect of higher intermolecular interaction, between phenolic compounds and protein films (Siripatrawan and Harte, 2012). There is evidence confirming the antibacterial activity of the zein film incorporated with 40 mg/ml Laurostearic acid, 0.0375 mg/ml nicin and 5058 mg/ml EDTA against *L. monocytogenes* and *S. enteritidis* (Hoffman et al., 2001). According to this study the number of *L. monocytogenes* declined from day 2 in plates containing zein films including Laurostearic acid or nicin whereas, the control film showed an increase. In another study Güçbilmez et al. (2007) found the effectiveness of zein film incorporated lysozyme, albumin protein and EDTA against *E. coli* and *Bacillus subtilis*. Del Nobile et al. (2008) found a significant difference between zein control films and zein films integrated thymol.

Water vapor transmission

The WVTR data showed that films prepared from ZEO revealed lower transmission rate than zein films. It appears that the essential oil of *Z. multiflora* containing hydrophobic antimicrobial compounds, could improve the moisture barrier properties (Moradi et al. 2012). According to Moradi et al. (2012) the chitosan films organized from ZEO showed lower transmission rate than chitosan film. In another study (Pintado et al., 2010) showed an increase of concentration of glycerol and sorbitol from 1.5 to 3.0% resulted in an increase in WVT. Hydroxyl groups of the plasticizers change polymer–polymer interactions by developing polymer-plasticizer hydrogen bonds, by this means increasing intermolecular spacing and transmission of film material (McHugh et al., 1994). According to Del Nobile et al. (2008) low gas permeability is frequently a great advantage in maintaining the quality of food products. This is classified with general preventative characteristics of plastics.

Conclusion

This study tries to use polymer material and natural antimicrobial agents for cheese packaging. All investigated features of ZEO formulated zein films were controlled by ZEO concentrations. WVT and antioxidant activities were improved after adding ZEO into zein film, while all films incorporated with antimicrobial agents showed lower strength and elongation values than the control films. These results might have application in cheese, which is effected by microbial spoilage. So, zein antimicrobial edible film might help the packaging system of the Iranian ultrafiltered Feta cheese. It could be concluded that plant extracts, with their powerful antimicrobial and antioxidant activity, may be the ideal biopreservative agents for cheese. The application of ZEO formulated zein films in cheese packaging was sufficient to prevent *S. enteritidis, L. monocytogenes, E. coli and S. aureus* growth and to conserve the overall quality and sensory attributes in treated cheese during 70 days storage. The proposed biopreservative agents incorporated in zein edible film could be innovatively applied for the preservation of dairy products.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Minimally processed fruit salad enriched with *Lactobacillus acidophilus*: Viability of anti-browning compounds in the preservation of color

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Minimal processing promotes browning of some vegetal tissues due to cell membrane disruption, which results in the release of oxidative enzymes. This study evaluated the efficiency of citric acid, ascorbic acid, sodium metabisulfite and L-cysteine hydrochloride to retard enzymatic browning of minimally processed fruit salad and enriched this product with *Lactobacillus acidophilus* LA-5. Control treatment was fruit salad immersed in water. Polyphenol oxidase (PPO) and color (L*, a*, b*, index color - CI, browning index - BI, c*, and h°) were analyzed. The viability of *L. acidophilus* was also evaluated using Rogosa agar in fruit salads containing anti-browning compounds in higher concentrations. PPO presented a significant difference among control and fruit salad treated with ascorbic acid and L-cysteine hydrochloride, indicating the highest anti-browning activity of these compounds. The fruit color was affected by processing and storage time, with a reduction in the values of L* over time. Values of a*, c*, h° angle and CI indicated a predominance of red color in the fruit salad. Salads containing anti-browning compounds in higher concentrations presented viability of *L. acidophilus* above 7.43 log CFU/g up to the fifth day of storage, indicating that the product can be promised as probiotic. Thus, the fruit salad treated with anti-browning compounds has potential use as a probiotic carrier.

Key words: Fresh-cut fruits, color, ascorbic acid, vegetable matrix, probiotic culture.

INTRODUCTION

In recent years, concerns about health and wellness have lead consumers to seek a healthy diet by eating more fruits. Then, a rapid market growth for fresh cut fruits and vegetables has been observed once consumers increased

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Abbreviations: PPO, Polyphenol oxidase; **PVPP,** polyvinylpolypyrrolidone; **CI,** color index browning; **BI,** index.

demand for high nutritive value, convenience and fresh-like quality (Rico et al., 2007; James and Ngarmsak, 2010). According to De Ancos et al. (2011), minimally processed fruits and vegetables are plant products that have been subjected to physical changes to maintain their freshness. Fruit mixes, in which two or more fruits are combined to obtain a product with special characteristics, are often used to meet consumer needs regarding flavor, texture and the nutritional value of fruit (Rojas-Graü et al., 2011). Some studies focus on the use of probiotic microorganisms in minimally processed fruit (Rößle et al., 2010a, b; Alegre et al., 2011) and in juices from fruits and vegetables (Yoon et al., 2004; Yoon et al., 2005; Yoon et al., 2006; Sheehan et al., 2007). These products have presented promising results and as such are considered promoters of health and wellness. Probiotic microorganisms benefit the host organism by balancing intestinal microbiota. Microorganisms of the genus *Lactobacillus* are among the most commonly used as probiotics in food applications (Soccol et al., 2010). Fermented milk products are generally good carrier matrixes for these microorganisms. However, other food matrixes have been studied as potential carriers for probiotic microorganisms. According to Soccol et al. (2010) and Vitali et al. (2012), raw and processed fruits may provide an ideal substrate for probiotic cultures since they contain essential nutrients for microorganism multiplication, such as minerals, vitamins, antioxidants and fibers. Furthermore, the use of fruit matrixes as probiotic carriers allows the consumer to choose probiotic foods free of cholesterol and lactose constituents present in dairy products.

However, a major problem with minimally processed fruits is their short shelf life, caused by enzymatic or microbial deterioration that causes undesirable visual effects on food (Rößle et al., 2009; Rojas-Graü et al., 2009). The process of peeling and cutting promotes the release of enzymes, causing the fruit surface to brown and negatively affecting product appearance (Ragaert et al., 2011). Thus, researchers are constantly looking for new ways to extend the shelf life of minimally processed fruits. Among these technologies, the use of anti-browning compounds, such as cysteine, ascorbic acid, citric acid and sulfite, which act to inhibit enzymatic browning, have emerged as potential alternatives (Baldwin and Bai, 2011).

In this context, some of the anti-browning agent, such as ascorbic acid acts as an oxygen scavenger and it is benefic to the probiotic food. Oxygen content and redox potential have been show to be important factors that contribute for the viability of probiotic bacteria during the shelf life of the food. According to Barbagallo et al. (2012), ascorbic acid acts as scavenger by removing the molecular oxygen from the reactions catalyzed by PPO and moreover it has a chelating effect on Cu present on prosthetic group of the polyhenol oxidase enzyme. So its action anti-browning promotes a favorable environment to the probiotic bacteria in fruits. Dave and Shah (1997) evaluated the viability of probiotic bacteria in yogurt supplemented with four levels of ascorbic acid and they verified that the addition of ascorbic acid favored the viability of *Lactobacillus delbrueckii* ssp. *bulgaricus*.

Therefore, considering the sensitivity of fruits to enzymatic browning and the positive effect that the anti-browning agents could promote on the viability of probiotic culture, this study aimed to evaluate the efficiency of citric acid, L-cysteine hydrochloride, ascorbic acid and sodium metabisulfite in minimally processed fruit salad enriched with *Lactobacillus acidophilus*, as well as to determine the viability of *L. acidophilus* in this minimally processed food product.

MATERIALS AND METHODS

Minimal processing of fruits, fruit salad preparation and inoculation with *L. acidophilus*

Pineapple, banana, guava, apple, papaya and mango were selected in the Rio Pomba, Minas Gerais, Brazil, market for fruit salad preparation. These fruits were obtained at ripening stage and sent to the Fruit and Vegetable Processing Unit at the Food Science and Technology Department at the Federal Institute of Education, Science and Technology of Southeast of Minas Gerais. Fruits were washed in tap water to eliminate dirt and impurities. Following this, fruits were immersed in water at 5°C with sodium dichloroisocyanurate (Sumaveg®, Diversey Lever) with an active residual chlorine concentration of 200 mg/L for 20 min. Afterward, fruits were peeled and manually cut using stainless steel knives. After cutting, fruit salads were prepared using the same proportion of fruits in each salad. Prepared fruit salads were immersed in a citric acid and sodium citrate buffer solution containing approximately 10^{10} CFU/mL of *L. acidophilus* LA-5 (Christian Hansen®). This strain is recognized as probiotic being widely used in the food industry. The probiotic culture was prepared and added to fruit salads according to Rößle et al. (2010a). Initially, the probiotic culture was activated twice in Man Rogosa Sharpe broth (MRS) and kept at 37°C for 18 h. The probiotic culture was then activated in MRS broth for 16 h, and centrifuged at 5°C for 15 min at 7000 *g* in a centrifuge model Sorvall Biofuge Stratos (Thermo Scientific). The broth supernatant was discarded and the obtained probiotic cell pellet was aseptically resuspended in a buffer consisting of a 1:1 ratio of citric acid and sodium citrate, pH 3.8 in order to wash cells. Then, it was centrifuged again in the same conditions. Afterwards, for each gram of probiotic cells it was added 10 mL of buffer solution of citric acid: sodium citrate (1:1, pH 3.8) to obtain at least 10^{10} cells/mL.

Finally, 1 mL of probiotic cell suspension was added to each gram of fruit salad to obtain this food containing *L. acidophilus*. The salads were in contact with the probiotic cell suspension for 15 min at 5°C. The control treatment was a minimally processed fruit salad without the addition of *L. acidophilus*.

Treatment of fruit salad with anti-browning compounds

To evaluate the effectiveness of the anti-browning compounds, salads containing *L. acidophilus* were immersed in 1 and 2% citric acid, 1 and 2% ascorbic acid, 0.01 and 0.03% sodium metabisulphite and 0.5 and 1% L-cysteine hydrochloride. Fruit salads were immersed for 3 min at 5°C. The control treatment was a fruit salad immersed in water. Fruit salads (1,000 g) were drained to remove excess solution, packed in polypropilene containers of 00 g and 2

stored at 8°C for further analysis at 0 hour and after 24, 72 and 120 h of processing. All experiments were done in three replicates.

Determination of polyphenol oxidase (PPO) activity

The PPO activity of the control (without probiotic and anti-browning compounds) and the fruit salad containing *L. acidophilus* that was previously immersed in the anti-browning compounds was determined at 0 h and after 24, 72 and 120 h of processing, according Teisson (1979). The extract was obtained by macerating 10.02 g of fruits (1.67 g of each one) with 0.5 g of polyvinyl-polypyrrolidone (PVPP), three drops of Triton X-100 and 20 mL of 0.05 M phosphate buffer (pH 7), kept refrigerated. The homogenate was immediately filtered through a cheese cloth and centrifuged for 10 min at 5000 *g* at 0°C in a centrifuge model Sorvall Biofuge Stratos (Thermo Scientific). The resulting supernatant was used to determine enzymatic activity. An aliquot of 1 mL of the obtained supernatant was added to 3.6 mL of 0.1 M phosphate buffer (pH 7) and 0.1 mL of 10 mM catechol that was left for 30 min in a water bath at 30°C. Afterward, the reaction was interrupted by adding 1.6 mL of 2 N perchloric acid. The enzymatic activity of polyphenol oxidase was expressed as units per gram of fresh weight per minute (U/g/min). One unit corresponded to the enzymatic activity capable of altering 0.001 absorbance at 395 nm.

Color determination

The surface color of the fruit salads was evaluated after inoculation with *L. acidophilus* and treatment with anti-browning compounds. The fruit surface color was measured using the colorimeter MiniScan EZ, HunterLab (Reston, Va., USA) at 0 h and after 24, 72 and 120 h of processing. Color determination was performed in reflectance mode by direct reading of the coordinates L*, a* and b*, using the CIELAB L* scale, where L* measures lightness and varies from 0 (black) to 100 (white), a* measures the tonality, from +a (red) to -a (green), and b* measures the saturation, from +b (yellow) to -b (blue). The color index (CI), which measures the color of the product, was determined according to Mazzuz (1996): CI = 1000 x a* / L* x b*. The browning index (BI) was determined according to Palou et al. (1999): BI = [100 (X-0.31)]/0.172, where X = (a* + 1.75L*) / (5.645L* + a* - 3.02b*). The color intensity or chroma (C*) and hue angle (h°) were calculated according to McGuire (1992): $c^* = [(a^*) + (b^*)]^{-1/2}$ and h° = arctan (b*/a*). To measure color, control and treated fruit salads were crushed and placed on a glass plate, and values of L*, a* and b* for each sample were read directly from the product. It was used at the same quantity of each fruit to measure the color.

Viability of *L. acidophilus* in minimally processed fruit salads

Samples of fruit salads (25 g) were homogenized in 225 mL of peptone saline solution (0.85% NaCl and 0.1% peptone). Serial dilutions were performed and cultured by the pour-plate method, in which 1 mL of the respective dilutions was added to a small amount of Rogosa SL agar (Himedia) in Petri dishes. Following this, the Petri dishes were kept in anaerobic jars at 37°C for 72 h. The viability of *L. acidophilus* was determined in the control treatment as well as in the fruit salads treated with anti-browning compounds at the highest concentrations (2% citric acid, 2% ascorbic acid, 0.03% sodium metabisulphite and 1% L-cysteine hydrochloride) at 0 h and after 120 h of fruit salad preparation in order to demonstrate that they do not inhibit the growth of probiotic bacteria. All fruit salads were stored at 8°C.

Statistical analyses

For the anti-browning compound experiments, we used a completely randomized statistical design using subdivided plots. This consisted of eight treatments and one control in the plots, with 3 replicates. Subplots were differentiated by assessments over storage time (0, 24, 72 and 120 h). The viability of *L. acidophilus* was evaluated using a completely randomized design with three replicates and a 4x2 factorial design. In this factorial design, four anti-browning compounds (1% L-cysteine hydrochloride, 2% ascorbic acid, 2% citric acid, 0.03% sodium metabisulfite) and two storage times (0 and 120 h) were evaluated. Analysis of variance (ANOVA) and the Dunnett's test for multiple comparisons among means were used to analyze the results, considering a 5% level of significance, using the R software (R Core Team, 2012).

RESULTS AND DISCUSSION

The color of the fruits present in the salad was affected by processing (Table 1) and storage time. The polyphenol oxidase activity presented significant differences ($p<0.05$) among the control treatment and fruit salads treated with 1 and 2% ascorbic acid and those treated with 0.5 and 1% L-cysteine hydrochloride. These results indicate that ascorbic acid and L-cysteine hydrochloride were the best browning inhibitors, since these treatments presented lower enzyme activity than the fruit salads treated with 0.01 and 0.03% sodium metabisulfite and those treated with 1 and 2% citric acid. Sodium metabisulfite and citric acid did not differ significantly from control treatment, presenting an average PPO activity value of 1.67 U/g/min (Table 1). However, we verified with the naked eye that apple and banana present in fruit salads treated with 0.5% L-cysteine hydrochloride showed undesirable pinkish surface coloration over storage time. Melo and Vilas Boas (2006) found that the lower the concentration of cysteine, the higher the pinkish intensity in minimally processed 'apple' banana. According to Richard-Forget et al. (1992), this is probably due to the regeneration of phenols when the applied amount of cysteine is low, which probably explains the pink coloration in apple and banana treated with only 0.5% of this anti-browning compound. The browning of vegetal tissues is due to disruption of the cell membrane during the processing, which promotes the release of enzymes that come in contact with the phenolic substrates, thus having an uncontrolled oxidation, using molecular oxygen (De Ancos et al., 2011), as we observed in this work. The lightness (L*) of fruit salads was not significantly different among treatments ($p>0.05$). However, storage time did have a significant effect ($p <0.05$) on this colorimetric parameter, as a result of the fruits salad browning over time (Figure 1A). Browning is an undesirable characteristic of minimally processed fruit. González-Aguilar et al. (2008) observed less browning in fresh cut mango treated with ascorbic acid, as we verified. Colorimetric parameters a* and b* did not present a significant difference ($p >0.05$) among treatments (Table 1).

However, storage time had a significant effect on these parameters (Figures 1B and 1C), occurring a increasing

Table 1. Mean values of polyphenol oxidase (PPO) activity (U/g/min) and color parameters (L*, a*, b*, CI, BI, c* and h°) of fruit salads treated with anti-browning compounds.

Treatment	PPO	L*	a*	b*	CI	BI	c*	h°
AA1	1.26[b]	57.60[a]	23.89[a]	33.51[a]	12.56[a]	112.66[a]	41.28[a]	1.26[a]
AA2	1.23[b]	57.46[a]	23.70[a]	32.33[a]	12.85[a]	108.57[a]	40.13[a]	1.24[a]
CC05	1.30[b]	57.18[a]	23.99[a]	32.45[a]	12.98[a]	110.05[a]	40.30[a]	1.23[a]
CC1	1.18[b]	59.04[a]	22.57[a]	33.43[a]	11.57[a]	107.12[a]	40.39[a]	1.34[a]
MS001	1.67[a]	57.64[a]	22.40[a]	35.87[a]	11.11[a]	119.41[a]	42.40[a]	1.44[a]
MS003	1.53[a]	58.65[a]	21.74[a]	34.66[a]	10.81[a]	111.27[a]	40.96[a]	1.44[a]
AC1	1.65[a]	58.89[a]	21.83[a]	36.85[a]	10.09[a]	119.13[a]	42.86[a]	1.50[a]
AC2	1.44[a]	58.78[a]	22.75[a]	37.35[a]	10.48[a]	122.10[a]	43.80[a]	1.46[a]
Cont.	1.67[a]	56.69[a]	22.92[a]	34.12[a]	12.07[a]	116.74[a]	41.18[a]	1.34[a]
DMS-Dunnett	0.26	2.57	2.49	5.36	1.99	20.72	4.96	0. 20

CI, color index; BI, browning index; AA1, 1% ascorbic acid; AA2, 2% ascorbic acid; CC05, 0.5% L-cysteine hydrochloride; CC1, 1% L-cysteine hydrochloride; MS001, 0.01% sodium metabisulphite; MS003, 0.03% sodium metabisulphite; AC1, 1% citric acid; AC2, 2% citric acid; Cont., Control. Mean values followed by the same letter in lowercase in the column have no significant difference at 5% of probability by Dunnett's test.

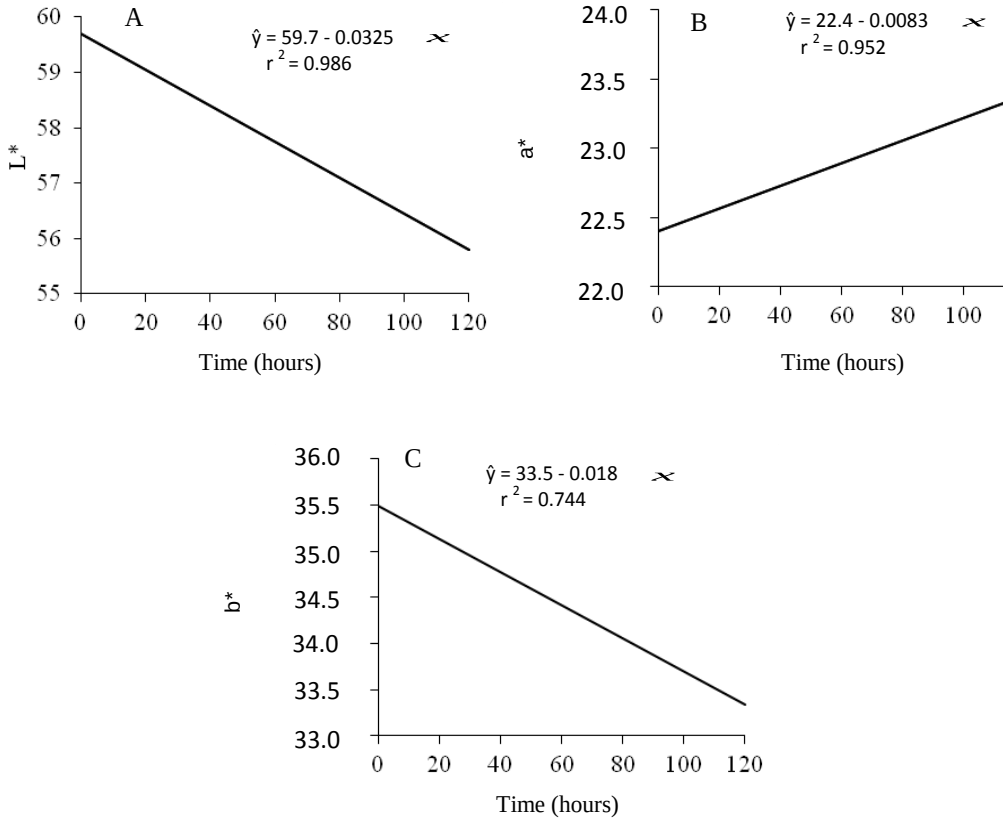

Figure 1. Regression equation and colorimetric coordinate determination coefficients L* (A), a* (B), and b* (C) of minimally processed fruit salads treated with anti-browning compounds for 3 min at 5°C and stored for 120 h.

in a* values which tend toward red, and a reduction of b* values, which tend toward blue, during storage time. Similar to our results, Pizato et al. (2013) observed increasing values of a* in minimally processed apple over

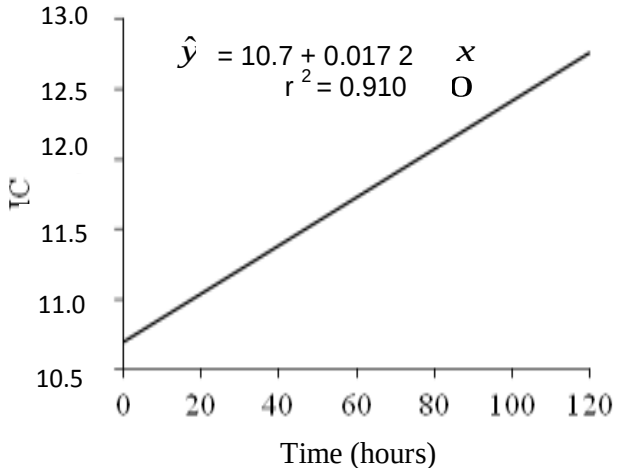

Figure 2. Regression equation and color index (CI) determination coefficient of minimally processed fruit salads treated with anti-browning compounds for 3 min at 5 °C and stored for 120 h.

storage time for the tested treatments. The color index (CI) ranges from -20 to +20, in which negative values indicate a greener fruit color and positive values indicate intense red coloration (Mazzuz, 1996). In the present study, fruit salads presented an increase in CI over storage time (*p<0.05*) (Figure 2). However, it was not observed significant differences (*p>0.05*) among treatments for this colorimetric parameter. The browning index (BI), which measures the purity of the brown color, increased over storage time in fruit salads at 8 °C (*p<0.10*). These results are in agreement with those of Javdani et al. (2013), who worked with minimally processed apple and observed increasing values of BI as a function of time. However, a significant difference among treatments was not observed (*p>0.05*) in this work (Table 1). Another colorimetric parameter assessed was the Chroma index (c*), which denotes the saturation or color intensity. Smaller values of c* correspond to weaker color patterns (matting color of an object), while higher values indicated stronger or brighter colors, with the latter being desirable in food products (Cardoso et al., 2007).

In this study, there was no significant difference among treatments (*p>0.05*) for this parameter, with an average c* value of 41.47 (Table 1). Values of c* close to zero indicate neutral colors (gray) and values around 60 indicate vivid colors. Thus, although the storage time negatively impacted the color intensity of fruit salads (*p<0.01*), these products retained brighter colors. Also, hue angle (h°) is a colorimetric parameter often used to express color variations in vegetable products (McGuire, 1992). Red is related to hº equal to zero, while yellow is related to hº equal to 90º, green to 180º and blue to 270º. Fruit salads presented hº values ranging from 1.23° to 1.50° (Table 1), located in the first quadrant. These results showed the predominance of red coloration in the

Table 2. Mean values of lactic acid bacteria count (Log CFU/g) in minimally processed fruit salad treated with anti-browning compounds, at 0 h and after 120 h of processing and storage at 8°C.

Time (h)	Treatment				Mean
	CC1	AA2	AC2	MS 003	
0	8.53	8.42	8.50	8.38	8.46[a]
120	7.61	7.61	7.42	7.32	7.49[b]
Mean	8.07[A]	8.01[A]	7.96[A]	7.85[A]	

CC1, 1% cysteine hydrochloride; AA2, 2% ascorbic acid; AC2, 2% citric acid; MS 003, 0.03% sodium metabisulphite. Mean values followed by the same uppercase letter on the line or lowercase letter in the column are not difference according to the F test at 5% probability.

salads. The analysis of *L. acidophilus* viability in minimally processed fruit salads showed that the concentration of this probiotic microorganism ranged from 8.38 Log CFU/g in the fruit salad treated with 0.03% sodium metabisulphite to 8.53 log CFU/g in the fruit salad treated with 1% L-cysteine hydrochloride immediately after processing (time 0). Moreover, the anti-browning compounds in the highest concentrations did not affect (*p>0.05*) the viability of *L. acidophilus* (Table 2) showing that they could promote a favorable environment to the probiotic bacteria in the salad. However, after 120 h of storage at 8°C, we observed a significant reduction (*p<0.05*) in the viability of *L. acidophilus*. There was no growth of this bacterium in the control treatment. Rößle et al. (2010a) used *L. rhamnosus* GG in minimally processed apples treated with the antioxidant Natureseal® AS1 and found that after 10 days of storage, the product contained 10^8 CFU/g of this microorganism.

Published works are not clear regarding the minimum concentration of probiotic microorganisms needed to promote beneficial effects on the host organism. Some researchers suggest concentrations greater than 10^6 CFU/g (Dave and Shah, 1997; Saad, 2006) while others suggest concentrations of at least 10^7-10^8 CFU/g (Lourens-Hattingh and Viljeon, 2001). Thus, based on reviewed literature and considering that the developed fruit salads treated with different anti-browning compounds contained over 10^7 CFU/g of *L. acidophilus*, the developed fruit salads have potential for use as probiotic carriers. This high count of *L. acidophilus* does not promote deterioration of the product because it is maintained on cooling. Thus, the population is only viable, as found, once the salad is not stored in the optimum temperature for growth of *L. acidophilus*.

Conclusion

Ascorbic acid and L-cysteine hydrochloride were more effective in retarding the enzymatic browning of fruit

salads. However, we suggest the use of ascorbic acid due to its low cost. Also, ascorbic acid is an innocuous and natural product that does not promote undesirable color changes in the product. Fruit salads presented brown color over storage time at 8°C, which was confirmed by the reduction of L* values. Moreover, values of a*, c*, h° and CI indicated that the fruit salad color tended toward red with brighter colors being predominant. Anti-browning compounds did not have a negative impact on the viability of *L. acidophilus* and this fruit salad can be used as a promising probiotic carrier. Therefore, minimally processed fruit salads treated with anti-browning compounds and enriched with *L. acidophilus* constitute a promising functional probiotic product that can be consumed by vegetarians, children, elderly people and those with cholesterol restricted diets.

Conflict of interests

The authors did not declare any conflict of interest.

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Physico-chemical and rheological properties of prato cheese during ripening

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The influence of storage temperature (6, 12 and 18°C) on texture parameters of Prato cheese were evaluated during 22 days of ripening. A reduction of firmness, gumminess and elasticity was observed; however, cohesiveness and adhesiveness increased. With the increasing temperature, firmness and gumminess reduced, whereas the elasticity, cohesiveness and adhesiveness did not change. It can be concluded that storage temperature influences the texture profile of Prato cheese which present differences in the evaluated parameters during 22 days of ripening.

Key words: Cheese ripening, hard cheese, colour, texture profile analysis.

INTRODUCTION

Prato cheese is the cheese of semi-cooked dough, and it began to be manufactured in Brazil in the 1920s by Danish immigrants in an attempt to produce cheeses similar to Danish Danbo and Dutch Gouda cheeses (Furtado et al., 1994). Prato is a maturated cheese with average moisture, is semi-rigid, and exhibits a mild flavour and yellow hay colour provided by the natural dye urucum (Kubo et al., 2013). Prato cheese is produced in all regions of the country, with an average annual production of approximately 150,000 tons (ABIQ, 2010). Maintenance of the identity and quality standards of Prato cheese is of fundamental importance to meet the expectations of the consumer. Texture is an important parameter for the identification of quality, as the consumer usually evaluates the colour and aroma prior to other characteristics (Santos Júnior et al., 2012). Many parameters influence the texture of cheeses because the lower the fat content of cheese is, the denser the casein network and the firmer the cheese (Tunick et al., 1993).

Rheological methods that study material deformation and flow are used to measure the texture properties of materials. Furthermore, these methods are used to determine the elastic and viscous components as well as to elucidate short-reach interactions, such as casein conformation and structure (Steffe, 1996). One of the methods used to evaluate the texture of cheeses is a texture profile analysis (TPA), which simulates the compression of molar teeth during mastication, allowing

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the prediction of texture characteristics even before product consumption (Delgado et al., 2011). A TPA generates values of firmness, cohesiveness, elasticity, adhesiveness and gumminess that enable the definition of the texture parameters of cheese (Amar and Surono, 2012).

The objective of this study was to evaluate the physical and chemical characteristics of the cheese plate, in addition to studying the variations of texture of Prato cheeses by TPA at different temperatures (6, 12 and 18°C) at the 1st, 8th, 15th and 22nd day of maturation.

MATERIALS AND METHODS

The study was developed during the period from June 9th to July 3rd of 2014, in a dairy industry facility located in the city of Rio Verde, GO, Brazil, and the Post-Harvest of Vegetable, Fruits, Greenery and Products of Animal Origin Laboratories of the Federal Institute of Goiás, Rio Verde Campus, GO, Brazil. Prato cheeses were used that were produced by a dairy industry facility registered at the Federal Inspection Service of the Ministry of Agriculture, Animal Farming and Supply of Brazil.

Quality of refrigerated milk used to process prato cheese

The quality evaluation of the milk used to process the Prato cheeses was performed in the Milk Quality Laboratory from the Centre for Food Research of the School of Veterinary and Animal Science (Centro de Pesquisa em Alimentos da Escola de Veterinária e Zootecnia) of the Federal University of Goiás, Goiânia, GO, Brazil. Prior to cheese fabrication, milk samples were packaged in flasks containing Bronopol® preservative. After the collection of milk samples, the flasks were transported in isothermal boxes with ice for the evaluation of fat, protein, lactose, total dry matter and degreased dry extract contents using Milkoscan 4000 equipment (Foss Electric A/S. Hillerod, Denmark) (International Dairy Federation, 2000). Before the analysis, the flasks containing the milk samples were heated in a water bath at 40°C for 15 min for fat dissolution. The results are expressed in percentage (%).

Preparation of prato cheese

The Prato cheeses were produced with refrigerated milk, standardised at 3.3% fat, pasteurised under constant agitation at 75°C for 16 s, followed by cooling at the temperature of 35°C. Next, lactic acid cultures were added (mesophilic cultivation based on *Lactococcus lactis* and *Lactococcus* subsp. *cremoris* and thermophilic cultivation based on *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *L. delbrueckii* subsp. *helveticus*) at a proportion of 100 g of lactic acid cultures to 5,000 L of milk, calcium chloride (solution at 40% m/m) at a proportion of 2.5 to 5,000 L of milk, urucum natural dye (500 mL to 5,000 L of milk) and industrial rennet (300 mL for 5,000 L). Next, the milk was allowed to rest for 40 min until complete coagulation. Then, the cheese curd was cut using a 2 cm blade curd knife and allowed to rest for 10 min, followed by the removal of 30% of the milk whey. The second mass mixing took place 20 min afterwards, followed by pre-pressing in a pneumatic press and moulding in 4 kg moulds. After moulding in dewheying moulds, the Prato cheese was pressed for 120 min in a pneumatic press at 40 lb/pol², and the cheeses were immersed in brine at 10°C (21% of salt and pH 5.3) for 24 h. After brining, the cheeses were remained in the drying chamber for 24 h at 8°C, were packaged in thermo-shrinkable packaging and were stored in a BOD incubator oven at 10°C for maturation (for 22 days).

Physical and chemical analysis of prato cheeses

Instrumental analysis of the texture profile of prato cheese

The instrumental texture of Prato cheese was measured over 22 days (1st, 8th, 15th and 22nd day of maturation). Thirty-six cheese bars of ± 4 kg, divided into three equal parts to improve sampling, were used. A 2 cm piece of the outer layer of cheese (crust) was discarded so as not to interfere with the analysis. The cheese was cut in 2 cm cubes with a calliper. Prior to the analysis of texture, the 2 cm Prato cheese cubes were conditioned at three temperatures (6, 12 and 18°C). During the maturation period, three repetitions were performed for each evaluation temperature, for the three sections of the cheese, giving a total of nine repetitions per temperature. The TPA was analysed using a Brookfield texturometer and a 5 g trigger, probe displacement speed of 1 mm/s and compression rate of 50%. The firmness, elasticity, adhesiveness, gumminess and cohesiveness parameters were obtained.

Instrumental colour parameters of prato cheese

The colour was analysed with a Hunter Lab digital colourimeter during the Prato cheese maturation period. For this step, the cheese was cut into 2 cm cubes, and cubes from the middle part and extremities of the cheese were selected to provide better sampling. The cubes were placed on the reader to obtain L*, a* and b* values, which were converted into Chroma ($[(a * 2 + b * 2)^{1/2}]$) and Hue ($[(b * / a *) \tan^{-1}]$) (Venturini et al., 2011). Sampling was performed as described by the TPA, giving a total of 27 colour analyses per day of evaluation.

Physico-chemical parameters of prato cheese

The pH, titratable acidity and moisture of Prato cheese were analysed according to the methodology proposed by the AOAC (1995) during the maturation period. The fat content was analysed by Gerber's method (Furtado, 1975), the total protein by Kjeldahl's method (AOAC, 1995) and the ash content by the gravimetric method (AOAC, 1995) at the 22nd maturation day.

Statistical analysis

The statistical analysis was performed using a completely randomised design with Tukey's test for the comparison of textures, at the temperatures of 6, 12 and 18°C, between the 1st, 8th, 15th and 22nd maturation days. The average results of pH, titratable acidity and moisture between the maturation days were compared by Tukey's test. For the statistical analysis, a Sisvar computer program (Ferreira, 2008) was used. Differences between treatments were considered significant at $p<0.05$. The average fat, protein and ash results were presented descriptively' from the statistical analysis.

RESULTS AND DISCUSSION

The milk used in the preparation of Prato cheeses presented average values of 3.67 ± 0.01% fat, 3.17 ± 0.01% protein, 4.32 ± 0.01% lactose, 12.12% ± 0.01% total dry matter and 8.45 ± 0.02% non-fat dry matter. These values are considered adequate for industrial cheese processing in Brazil, where the legislation determines a minimum content of 3.0% fat, 2.9% total protein, 11.4% total dry matter and 8.4% degreased dry

Table 1. Firmness (g) of Prato cheese at 6, 12 and 18ºC during the maturation period.

Storage (days)	Temperature		
	6ºC	12ºC	18ºC
1	10603.67±789.72[a]	9281.11±530.83[a]	7852.67±487.06[a]
8	9860.89±498.22[a]	7974.66±1065.25[ab]	7684.56±246.89[a]
15	7928.50±374.08[b]	7401.22±1204.25[b]	6512.33±234.06[b]
22	7691.00±563.56[b]	6599.55±640.43[c]	6344.00±388.53[b]
CV (%)	6.41	11.58	5.09

Averages in the columns followed by different letters represent significant differences according to Tukey's test at 5% significance.

Table 2. Cohesiveness of prato cheese evaluated at 6, 12 and 18°C during the maturation period.

Storage (days)	Temperature		
	6ºC	12ºC	18ºC
1	0.62±0.02[b]	0.65±0.02[a]	0.63±0.02[b]
8	0.63±0.02[ab]	0.65±0.02[a]	0.65±0.01[ab]
15	0.65±0.02[a]	0.66±0.02[a]	0.65±0.02[ab]
22	0.65±0.02[a]	0.66±0.01[a]	0.66±0.02[a]
CV (%)	2.79	2.47	2.33

Averages in the columns followed by different letters represent significant differences according to Tukey's test at 5% significance.

Table 3. Adhesiveness (mj) of Prato cheese evaluated at 6, 12 and 18°C during the maturation period.

Storage (days)	Temperature		
	6ºC	12ºC	18ºC
1	0.82±0.04[a]	0.79±0.03[a]	0.79±0.06[a]
8	0.83±0.01[a]	0.83±0.10[a]	0.79±0.03[a]
15	0.84±0.05a	0.84±0.05[a]	0.80±0.07[a]
22	0.85±0.05[a]	0.86±0.05[a]	0.84±0.05[a]
CV (%)	5.96	7.77	6.94

Averages in the columns followed by different letters represent significant differences according to Tukey's test at 5% significance.

extract (Brasil, 2011). There are no current standards for lactose. During the maturation period, the firmness of Prato cheese evaluated at the temperatures 6, 12 and 18°C decreased with increasing temperature (Table 1).

The cohesiveness of Prato cheese evaluated at the temperatures of 6 and 18°C increased during the maturation period; however, at 12°C, it remained constant (Table 2). The adhesiveness of Prato cheese evaluated at the temperatures 6, 12 and 18°C remained constant during the maturation period (Table 3). The elasticity of Prato cheese evaluated at 6, 12 and 18°C Table 4. Elasticity (mm) of Prato cheese evaluated at the temperatures 6, 12 and 18°C during the maturation period. decreased during the maturation process (Table 4). The gumminess of Prato cheese evaluated at 6, 12 and 18°C decreased during the maturation period (Table 5). Regardless of the different evaluation temperatures, during the maturation period, the Prato cheese had reduced firmness and increased cohesiveness, and the adhesiveness remained constant. The same pattern was reported by Sant'ana et al. (2013) for the texture evaluation of fresh Minas cheese during 21 days of storage. Guinee et al. (2001) also reported that the firmness of Mozzarella cheese was reduced during maturation. At the 8th day of maturation at 18°C, Prato cheese presented a firmness of 7,684.56 g, a cohesiveness of 0.65 and an elasticity equal to 8.44 mm. Instrumental texture values lower than the values obtained in the present study were reported by Trancoso-Reyes et al. (2014), who evaluated the texture of traditional Mexican cheese 10 days after production. They verified a firmness of 5,810 g, a cohesiveness of 0.22 and an elasticity of 4.5 mm. At the 22nd maturation day, Prato cheese evaluated at 6°C presented a firmness of 7,691 g. Higher firmness values than those obtained for the present study were reported by Bayarri et al. (2012), who evaluated the viscoelasticity and texture of cream cheese refrigerated at 10°C. These authors reported firmness values of 13,054 g.

Storage (days)	Temperature		
	6ºC	12ºC	18ºC
1	8.58 ±0.03[a]	8.69 ±0.14[a]	8.45 ±0.21[a]
8	8.47 ±0.03[a]	8.35 ±0.17[b]	8.44 ±0.21[a]
15	8.18 ±0.08[b]	8.25 ±0.10[b]	8.25 ±0.10[ab]
22	8.16 ±0.15[b]	8.01 ±0.22[c]	8.11 ±0.12[b]
CV (%)	1.93	1.95	2.04

Averages in the columns followed by different letters represent significant differences according to Tukey's test at 5% significance.

The Prato cheese in the present study presented constant firmness during the first eight days of evaluation; according to De Jong et al. (1976) during this phase,

Table 5. Gumminess (g) of Prato cheese evaluated at 6, 12 and 18°C during the maturation period.

Storage (days)	Temperature		
	6ºC	12ºC	18ºC
1	6971.67±652.12[a]	6257.78±398.13[a]	5049.56±502.41[a]
8	6368.00±368.85[a]	5073.33±575.33[b]	4851.00±677.13[ab]
15	5096.63±443.78[b]	4851.00±677.13[bc]	4795.78±518.81[ab]
22	4737.88±552.82[b]	4193.33±371.44[c]	4193.33±371.44[b]
CV (%)	8.84	10.23	11.20

Averages in the columns followed by different letters represent significant differences according to Tukey's test at 5% significance.

Table 6. Firmness (g), cohesiveness, adhesiveness (mj), elasticity (mm), and gumminess (g) of Prato cheese at the 22nd maturation day at different temperatures.

Texture	Temperature (ºC)			CV (%)
	6%	12%	18%	
Firmness	10603.67±789.72[a]	9281.11±530.83[b]	7852.67±487.06[b]	7.89
Cohesiveness	0.65±0.02[a]	0.66±0.01[a]	0.66±0.02[a]	2.51
Adhesiveness	0.85±0.05[a]	0.86±0.05[a]	0.84±0.05[a]	6.23
Elasticity	8.16±0.15[a]	8.01±0.22[a]	8.11±0.12[a]	2.09
Gumminess	4737.88±552.82[a]	4193.33±371.44[b]	4193.33±371.44[b]	9.97

Averages in the same row followed by different letters represent significant differences according to Tukey's test at 5% significance.

there are no significant alterations in cheese texture, which is the result of degradation at the protein matrix that occurs after the lipid phase, a discontinuous process. From the 8th to 15th day of maturation, Prato cheese had reduced firmness. According to De Jong et al. (1976), in the first seven to 14 days of maturation, rubbery cheese texture converts into the characteristics of the variety under analysis. It is believed that during this phase, proteolysis of the casein network stops and hydrolysis by residual coagulation of approximately 20% of casein-αs1 starts, producing peptide αs1-I and causing weakening of the casein network. At the maturation period from the 15th to 22nd day, the reduction of firmness in Prato cheese was noticeable. De Jong et al. (1976) reported that after 14 days of maturation, gradual texture changes occur in cheese. It is during this time period that the remaining αs1-casein and other caseins are hydrolysed. The cohesiveness of Prato cheese during the maturation period remained constant. According to Bourne (2002), this occurs when the proportion of the compression area of the second area relative to the first area is the same in the TPA analysis.

During the TPA analysis, Prato cheese presented high elasticity, almost returning to the same initial state when it is compressed 50%. Creamer and Olson (1982) observed that cheeses with higher pH are plastic and elastic. At a higher pH, the casein molecules have a negative charge, causing the protein aggregates to absorb water to solvate the non-neutral ionic charges. The elastic characteristic demonstrated by Prato cheese in the present study can be explained by the high content of fat (28.43%). According to Adda et al. (1982), cheeses of higher fat content are firmer and more elastic. The gumminess of Prato cheese decreased in the maturation period. According to Bourne (2002), gumminess is directly proportional to firmness, and therefore, gumminess decreases with reduced firmness. This pattern was observed in the present study. At the end of Prato cheese maturation (22 days), firmness and gumminess were reduced with the increase in temperature up to 12°C, whereas elasticity, cohesiveness and adhesiveness were not influenced by the different temperatures at the moment of analysis (Table 6).

Hydrophobic interactions played an important role in the determination of the conformation and interaction of the protein molecules (Nakai and Li-Chan, 1988). They manifested as strong attraction forces between the non-polar groups separated by water (Ben-Naim, 1980). When dealing with molecule arrangement, the molecules are grouped as a function of temperature. At low temperatures, the molecules of a material do not move or move too little, as the result of low energy. Thus, the molecules move closer. However, at high temperatures, the molecules move further apart, and because of the

Table 7. Colour parameters of Prato cheese evaluated during 22 days of maturation.

Colour parameters	Storage (days)				CV (%)
	1	8	15	22	
Chroma	22.65±0.75^{d}	24.47±0.57^{c}	27.82±0.31^{b}	30.42±1.22^{a}	2.91
Hº	1.42±0.01^{c}	1.44±0.00^{b}	1.45±0.01^{b}	1.47±0.01^{a}	0.42

Table 8. The pH, titratable acidity (5 of lactic acid) and moisture (%) of Prato cheese during maturation.

Parameter	Storage (days)				CV (%)
	1	8	15	22	
pH	5.32±0.01^{a}	5.30±0.02^{b}	5.16±0.01^{c}	5.10±0.02^{d}	0.31
Titratable acidity	0.94±0.10^{c}	1.01±0.16^{c}	1.40±0.15^{b}	1.71±0.37^{a}	17.97
Moisture	43.61±1.24^{a}	43.60±1.23^{a}	43.65±1.24^{a}	43.61±1.24^{a}	2.85

Averages in the same row followed by different letters represent significant differences according to Tukey's test at 5% significance.

high energy, the molecules are in constant movement and need more space to be organised, a relation that is visible in the characteristics of water. In water at a solid state, the molecules contain lower energy and are closer to each other compared to those of water at a gas state (Malenkov, 2009). In the present study, the lower the temperature is, the higher the firmness of cheese, which can be explained by the closer proximity of molecules caused by the low molecular energy at colder temperatures. The same viscoelastic pattern was reported by Bayarri et al. (2012). When evaluating cheese at temperatures of 10 and 22°C, they reported that at lower temperatures, the samples presented higher firmness and rigidity values. The researchers attributed this factor to the formation of fat as a function of the evaluation temperature. By studying the effects of temperature on the viscoelasticity of cream cheeses, Brighenti et al. (2008) reported that the lower the temperature is at the moment of analysis, the higher the firmness of the product. During the maturation period, the colour parameters of Prato cheese (Chroma and H°) increased with the maturation time (Table 7). Averages in the same row followed by different letters represent significant differences according to Tukey's test at 5% significance. The colour parameters of Prato cheese, Chroma and Hº, increased during the maturation period. The same behaviour was observed by Buffa et al. (2001) in the evaluation of cheese colour during the maturation period. In turn, when evaluating the colour of Asiago cheese during the maturation period, Marchesini et al. (2009) reported the reduction of Chroma values and the increase of Hº. According to Nollet and Toldrá (2010), lipid and protein degradation causes colour changes in products.

The pH decreased and the titratable acidity increased during the maturation period of Prato cheese; however, the moisture remained constant (Table 8). At the 8th day of storage, Prato cheese presented a pH of 5.30, an acidity of 1.01% of lactic acid, and a moisture of 43.60%. Spadoti et al. (2005) reported values lower than those of the present study in the evaluation of Prato cheese at the 10th day of maturation, with a pH value of 5.12, an acidity of 0.97% lactic acid, and a moisture of 41.70%; the cheese was characterised as average moisture. The relationship between pH and acidity was inversely proportional in the present study. While the pH decreased, acidity increased; the same pattern was observed by Sauer-Leal and Okada (2008), who evaluated the physico-chemical characterisation of Prato cheese by infrared spectroscopy. According to Fox (1993), during cheese maturation, the pH is reduced because lactic acid is produced by microorganisms responsible for fermentation. In turn, by evaluating the effect of pH on Mozzarella cheese, Guine et al. (2002) reported an increase during the storage period. This pH increase is associated with the reduction of the lactate:protein ratio (Fox and Wallace, 1997); when this ratio decreases, curd loses its buffering capability (Czulak et al., 1969).

The moisture remained constant during the maturation period because the thermo-shrinkable packaging prevented moisture loss or gain from the environment. Moisture values higher than those of the present study were reported by De Rensis et al. (2009), who evaluated the physico-chemical, rheological and sensorial characterisation of Prato cheese, with 50.73% moisture. In turn, Roig et al. (2003) reported a moisture of 48.4% for the evaluation of Prato cheese obtained by ultrafiltration. The fat content of Prato cheese at the end of maturation was on average 28.43±0.79% in wet basis

and 50.4±1.34% fat in the dry extract, which is characterised as fat cheese. By analysing Prato cheese obtained by ultrafiltration, Roig et al. (2003) reported values for fat in wet basis and dry extract similar to those of the present study. Values lower than those of the present study were found by Spadoti et al. (2005), who evaluated Prato cheese at the 10th day of maturation, with fat values of 25.33%. A fat value slightly higher than that of the present study was reported by Sauer-Leal and Okada (2008), who evaluated the physico-chemical characterisation of Prato cheese by infrared spectroscopy, with an average of 28.68%.

The protein content of Prato cheese evaluated 22 days after maturation was on average 24.06±0.98%. A protein content similar to that of the present study was reported by Sauer-Leal and Okada (2008). Values lower than those of the present study were found by Vianna et al. (2008) and Roig et al. (2003), with protein contents of 22.7 and 18.42%, respectively. The ash content of Prato cheese at the end of maturation presented an average of 3.11±0.67%; higher values were reported by Sauer-Leal and Okada (2008) and Alves et al. (2013), who evaluated the physico-chemical characterisation of Prato cheese, with averages of 4.04 and 4.03%, respectively. Evaluating which parameters influence the texture of Prato cheese is of fundamental importance to establishing patterns that meet the expectations of consumers. Knowing that the packaging temperature of Prato cheese influences the texture parameters, it is necessary to determine the ideal packaging temperature to perform a texture analysis of cheese. According to this study, a temperature of 12°C is indicated because at this temperature, the cheese presents good firmness and is within the limits of storage temperatures.

Conclusions

During the maturation period of Prato cheese, a reduction of firmness, gumminess and elasticity was observed; however, cohesiveness and adhesiveness increased. With an increasing temperature of evaluation, Prato cheese had reduced firmness and gumminess, whereas the elasticity, cohesiveness and adhesiveness did not change. The pH of Prato cheese decreased as the maturation stage advanced, acidity increased, and the moisture remained stable. At the end of maturation, Prato cheese was characterised as fat cheese, with protein and ash contents compatible to those of other studies. According to the present study, the temperature at the moment of analysis influences the texture parameters of Prato cheese.

Conflict of interests

The authors did not declare any conflict of interest.

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Evaluation of the postharvest quality of Cagaita fruits (*Eugenia dysenterica* DC.) coated with chitosan and associated with refrigeration

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Cagaita fruits are subject to seasonality and perishability. This work aims to use scanning electron microscopy (SEM) to evaluate the physicochemical characteristics, texture, color and physical structure of cagaita fruits coated with different chitosan concentrations. The fruits were divided as follows: T0 (uncoated fruits), T1 (fruits coated with 1% (v/v) chitosan), T2 (fruits coated with 2% (v/v) chitosan) and T3 (fruits coated with 3% (v/v) chitosan). They were analyzed at 0, 10, 20 and 30 days of storage. Titratable acidity and soluble solids content showed no conservation of fruit characteristics; they showed better results for uncoated fruits, as well as weight loss, vitamin C and peak strain. The color of cagaita fruits confirmed ripening during storage regardless of treatment. Scanning electron microscopy showed that the film solution did not adhere, as desired, to the cell wall of fruits. As the results of fruits coated with 3% pectin were close to control, further studies should be carried out with higher coating percentages so that the fruit quality is maintained during storage.

Key words: Physical structure, film solution, quality, shelf life.

INTRODUCTION

Cerrado is the second largest biome in South America occupying an area of 25% of the Brazilian territory. It has extremely rich flora, averaging 1000 species of trees, 3,000 species of herbs and shrubs and 500 vines (Roesler et al., 2007). Due to the heterogeneity of the plant, some fruits of Cerrado are little studied, despite

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Abbreviations: SEM, Scanning electron microscopy, **DPIP,** 2,6-dichlorophenol indophenols sodium.

having great economic potential and nutrition (Santos et al., 2012). Cagaita (*Eugenia dysenterica* DC.), a typical fruit of the Brazilian savanna, has fragile shell, green and light yellow color , juicy pulp, pleasant flavor, but slightly acidic (Santos et al., 2012). The fruit has important nutritional characteristics represented by vitamin C, as well as polyunsaturated fatty acids such as linoleic acid, with contents higher than those found in coconut and olive oils and linolenic acid, with contents higher than those found in sunflower, soybean and peanut (Nietsche et al., 2004). Being a regional fruit that is subject to seasonality and due to few studies conducted to increase its shelf life, cagaita is not available for consumption throughout Brazil. However, whenever available, it is consumed fresh or incorporated into food products such as sweets, juices, liqueurs and jellies (Santos et al., 2012).

In search of new technologies to preserve the quality of cagaita fruits, structural, sensory and nutritional changes should be minimize through physical, chemical and gaseous treatments that can be applied to the product (Mahajan et al., 2014). One of these applications relates to the use of edible coating combined with modified atmosphere and/or temperature control (Maftoonazad et al., 2007). Such edible coatings help to reduce oxygen availability, breathing, water loss and oxidation reaction rate (Kerdchoechuen et al., 2011). Chitosan is a biopolymer that can be used as edible coating because of its nontoxic characteristics; it forms biodegradable films and prevents microbial activity (Soares et al., 2011). In the post-harvest quality, chitosan is responsible for reducing breathing rate, ethylene production and transpiration of plants, and contributes to the fungicidal properties of fruits (Luvielmo and Lamas, 2012).

This work aimed to evaluate the physicochemical characteristics, texture, color and physical structure of cagaita fruits coated with different chitosan concentrations by scanning electron microscopy (SEM).

MATERIALS AND METHODS

Cagaita fruits were collected at Fazenda Gameleira, municipality of Montes Claros de Goiás - GO (16°06'20 "S and 51º17'11" W), 592 m above sea level on September 14, 2014; they were subsequently transported to the Laboratório de Frutas e Hortaliças, Instituto Federal Goiano - Rio Verde Campus. Initially, fruits were selected by size, color and absence of mechanical damage and surface stains. Subsequently, they were sanitized with chlorinated water at 150 ppm for 15 min and dried at room temperature. Then, fruits underwent four treatments as follows: control treatment, T0 (fruit receiving no chitosan coating), T1 (fruits coated with 1% (v / v) chitosan), T2 (fruits coated with 2% (v / v) chitosan) and T3 (fruits coated with 3% (v / v) chitosan). The preparation of film solutions was carried out from chitosan solubilization in glacial acetic acid and water (800 ml of water and 50 ml of glacial acetic acid); pH was adjusted to 4.00, with sodium hydroxide solution (0.1 mol/L). For the distinct treatments, the following amounts of chitosan were added: 0 g (control, T0); 5 g (treatment 1); 10 g (treatment T2); 20 g (treatment T3). Subsequently, fruits were immersed in chitosan solution for about 1 min and allowed to dry naturally. Treatments were placed in Styrofoam trays with dimensions of 150 × 150 × 18 mm. Trays were placed in BOD with controlled temperature at 12°C ± 0.1. They were evaluated at 0, 10, 20 and 30 days in three replicates with six fruits each. In each day of analysis, 72 fruits were evaluated. In the three replicates, each repetition possessed six fruits in the tray. The following parameters were analyzed: weight loss, titratable acidity and soluble solids, ascorbic acid, texture (through peak strain), color and physical structure by scanning electron microscopy (SEM). Digital scale with accuracy of four places was used to measure weight loss, and the results were expressed as percentage of the original weight through the reserved lot for analysis, using the same fruits. The lot used for weight loss was separated from the lot used for physical and chemical analysis. The fruits of physical-chemical analysis were discarded each day of the analysis, since the lot used for weight loss was maintained until the 30 days analysis. That is, they were removed from the heavy weight, and BOD was stored again. Titratable acidity quantification was obtained by titrating the filtered juice with NaOH solution (0.01 N), and the results were expressed as % citric acid by method No. 986.13 (AOAC, 1992). The soluble solids content, expressed in °Brix, was evaluated by reading the juice in refractometer Atago N-2E according to AOAC standard No. 983.17 (1992). Ascorbic acid was determined by volumetric oxidation-reduction, titrating samples with a 2,6-dichlorophenol indophenols sodium (DPIP) solution by AOAC method No. 967.21 (2000). All reagents used were of Neon Comercial Ltda in São Paulo - SP - Brazil.

Texture, analyzed by the peak strain, was determined with the help of Brookfield texturometer, model CT3 texture analyzer. This technique consists of a uniaxial compression test at high deformation of samples using a cylindrical acrylic plate (model TA3/1000) at compression speed of 1 mm/s and 50% of sample deformation. The results are expressed in N/m^2. Color (L*, a*, b*) of cagaita fruits was analyzed in Hunter Lab colorimeter model Color Quest II. The evaluation used 10º observation angle and D65 as standard illuminant, which corresponds to natural daylight. The results were expressed as L*, a* and b* values, where L* (luminosity or brightness) values range from black (0) to white (100), a* values range from green (-60) to red (+60) and b* values range from blue (-60) to yellow (+60). The characterization of the physical structure of cagaita fruits consisted of removing the epicarp, drying at 60°C for 12 h and storing in desiccator. For scanning electron microscopy analysis (SEM), samples were placed on stabs, coated with a thin layer of gold and micrographed. Evaluation was performed at the multiuser Laboratório de microscopia de Alta Resolução at the Instituto de Física, Universidade Federal de Goiás using Scanning Electron Microscope, Jeol, JSM - 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging. Statistical analysis consisted of a 4 × 4 × 3 factorial design, with four treatments (control, 1, 2 and 3% chitosan), four storage times (0, 10, 20 and 30 days) and three replicates for each fruit tray analyzed. This results in nine replicates of each treatment studied by completely randomized design. The models were selected according to the determination coefficient and its significance was tested by the F test. The mean values for weight loss, titratable acidity, soluble solids, ascorbic acid, texture and color analyses were compared by the Tukey test at 5% probability with the help of the SISVAR software.

RESULTS AND DISCUSSION

Weight loss

Cagaita fruits showed similar average weight loss results (Figure 1). Throughout the storage period control fruits showed the lowest weight loss values. The fruits of

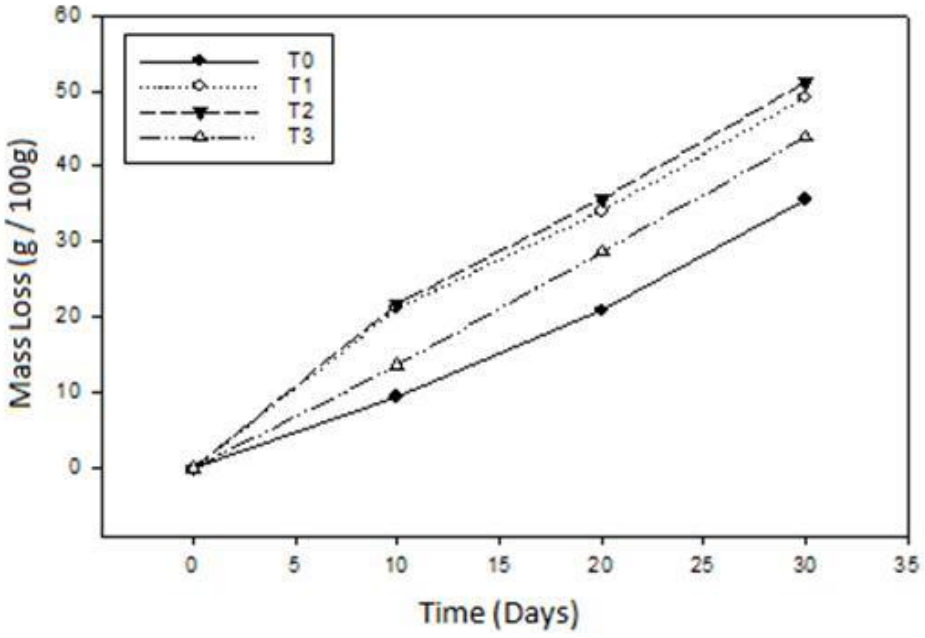

Figure 1. Weight loss for control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

treatment T2 (fruits coated with 2% chitosan) had the highest mass loss rate compared to the other treatments. Chitosan has an undesirable effect on the weight loss of cagaita fruits, where the smallest weight loss was observed in control fruits. However, fruits coated with 3% chitosan showed lower weight loss when compared to fruits coated with 1 and 2% chitosan (Figure 1). The non-protective effect of chitosan coating was also reported by Santos et al. (2008) in peach fruits. Although chitosan has the capacity of forming semi-permeable films that act by modifying the internal atmosphere and reducing loss by transpiration and dehydration (Santos et al., 2008), some authors report that hydrophilic molecules are not significant barriers to the dissemination of water vapor (Azeredo et al., 2010). Botrel et al. (2007) reported that components such as lipids and proteins contribute to decreased weight loss when added to coatings, since the matrix becomes more compacted with increasing amounts of amylase, thereby decreasing the coating permeability to water vapor. These authors reported that weight loss in coated garlic is due to the low barrier property of the coating to fruit transpiration and dehydration, as reported in this work. The smaller weight loss presented by fruits with 3% chitosan when compared to other treatments may be related to the coating thickness, because when too thin, coating can contribute to moisture loss (Silva et al., 2012).

Titratable acidity content

Titratable acidity of cagaita fruits varied during storage (Figure 2), showing lower values on the tenth day of analysis compared to the first day of storage and subsequent higher titratable acidity levels on the twentieth day analysis compared to the tenth day of storage. At the end of the experiment, fruits showed acidity levels similar to those reported on the tenth day of analysis. The drop in acidity levels on the tenth day of storage indicates that fruits are going through processes that lead to maturity, whereas according to Scalon et al. (2012), fruits with titratable acidity content above 1.5% are at the climacteric peak stage. Oshiro et al. (2012) reported that the drop in acidity content is a response to increased respiratory rate and contributes to water loss of fruits. On the twentieth day of storage, an unexpected increase of the titratable acidity content was observed for all treatments. This increase may be the result of anaerobic respiration that causes physiological disorders in fruits. The process of anaerobic respiration may indicate response to the low biofilm permeability to gases, causing the fruit to form ethanol and acetaldehyde by obtaining energy for this process, affecting the product quality (Steffens et al., 2007; Petracek et al., 2002). This increase can also occur through the release of galacturonic acid from the cell wall by the action of pectinmethylesterase and poligalac-turonase enzymes (Scalon et al., 2012). The decrease reported again on the thirtieth day of storage of cagaita fruits may be related to degradation caused by the anaerobic respiration phenomenon. Oshiro et al. (2012) reported that coating guavas with 3% chitosan does not contribute to the preservation of the titratable acidity content.

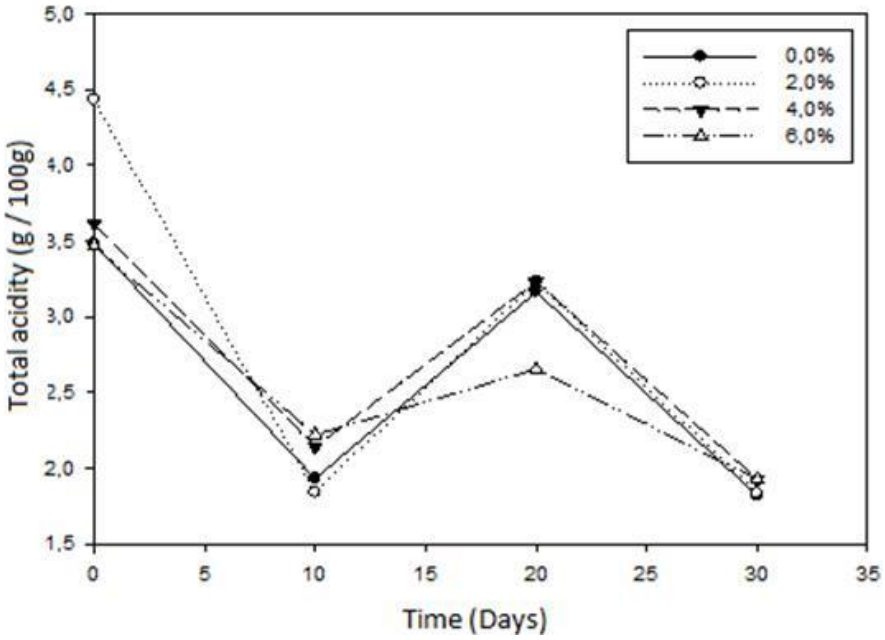

Figure 2. Titratable acidity of control fruits (T0), treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

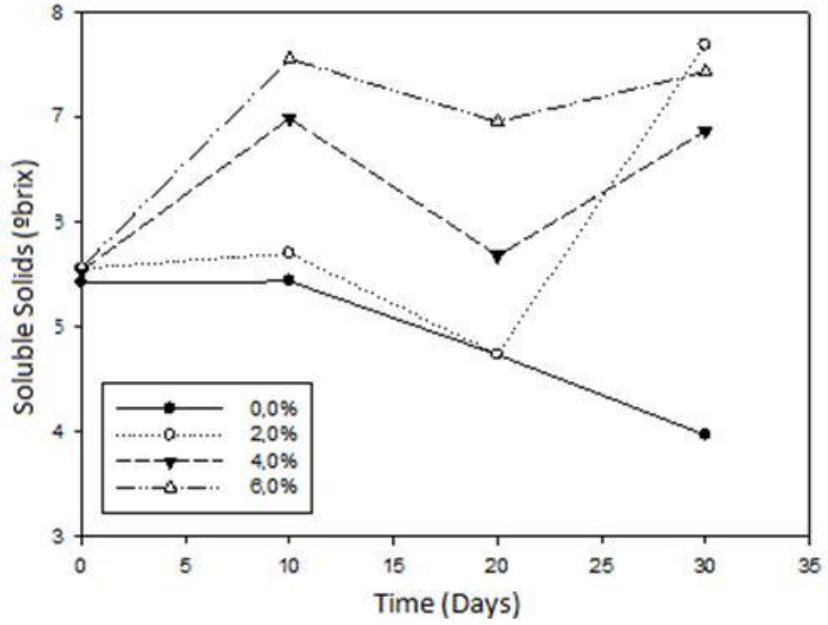

Figure 3. Soluble solids content of control fruit (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

Soluble solids content

Considerable variation in soluble solids content of uncoated and coated cagaita fruits was observed during storage (Figure 3). On the tenth day of analysis, fruits showed an increase in soluble solids content for treatments with 2 and 3% chitosan. On the twentieth day of analysis, decreases in the quantification of this parameter

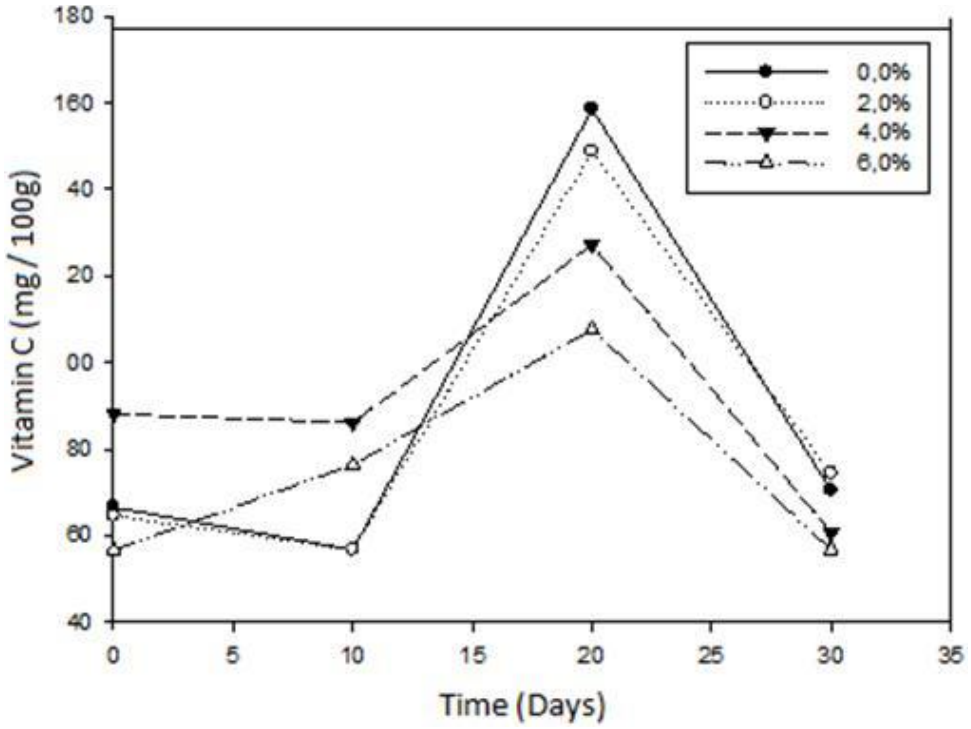

Figure 4. Vitamin C content of control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

for all treatments analyzed were observed, which, with the exception of uncoated fruits, increased again at the end of storage. The increase in the soluble solids content observed on the tenth and thirtieth day of analysis may be related to the characteristic of climacteric fruits, which even after harvest, still presents maturation evolution (Taiz and Zeiger, 2006). These fruits tend to increase the soluble solids content due to the biosynthesis of soluble sugars or degradation of polysaccharides (Kays, 1997). However, increase in soluble solids content may also indicate use of accumulated reserves in processes of fruit solid transformation into soluble sugars (Jeronimo and Kanesiro, 2000).

Santos et al. (2008) reported that in soluble solids analysis, an increase after a decrease may indicate concentration of solids due to loss of water during storage. On the twentieth day of storage analysis (Figures 2 and 3), with increasing acidity there was a decrease in soluble solids, which may indicate a rapid consumption of sugars through the aerobic respiration process. When this process is enhanced, future lack of sugar causes the onset of anaerobic respiration indicated by the increased sugar content and decrease in acidity values. Chien et al. (2013) determined the best results for preserving soluble solids in papaya fruits coated with 1% chitosan. Hong et al. (2012) showed a drastic increase in the soluble solids content of guavas coated with chitosan and control.

Ascorbic acid content

There were variations in the vitamin C content of stored cagaita fruits (Figure 4). At the tenth day of storage, except for fruits coated with 3% chitosan, there was a decrease in ascorbic acid content when compared to the beginning of the experiment. In twenty days of storage, fruits showed a considerable increase in vitamin C content, especially control fruits, and at the thirtieth day, the content showed a considerable drop. Reduced ascorbic acid content is related to the ripening of cagaita fruits at the tenth day of storage for fruits coated with 3% chitosan. Ripening was also analyzed for titratable acidity and soluble solids. Lee and Kader (2000) reported that, although the ascorbic acid content decreases with maturation, there may be an increasing trend due to the increase in the total volume of juice with advancing maturation process. This increase can be analyzed at the twentieth day of storage, but it is also the result of oxidative reactions that vitamin C undergoes during ripening by acting as a molecule with antioxidant properties (Carnelossi et al., 2009). Ascorbic acid has characteristics of unstable molecule that may undergo auto-oxidation into dehydroascorbic acid, which although being reversible, may cause losses in product quality (Gonçalves and Maia Campos, 2009) and lead to increased and decreased contents in stored fruits. This phenomenon can be caused by temperature used in the

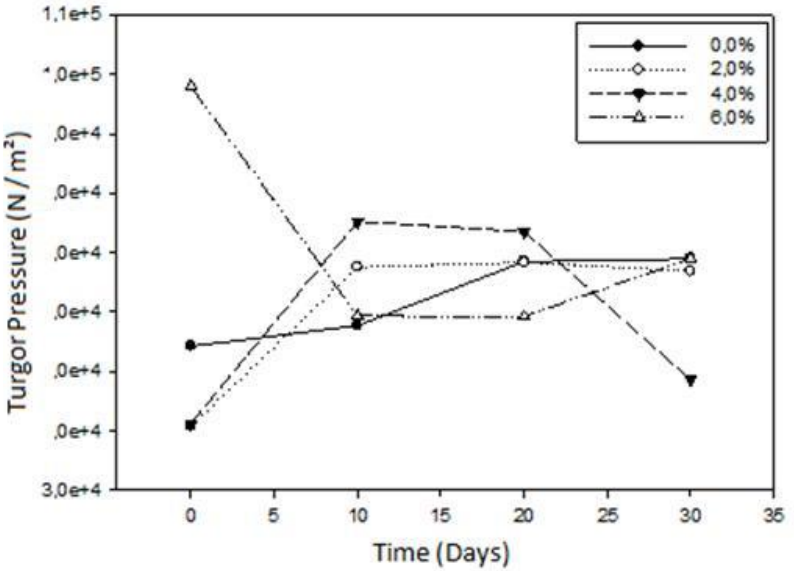

Figure 5. Peak strain of control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

experiment; in future work, a lower temperature storage can be used. At the end of the experiment, the decrease in ascorbic acid content can be related to chemical and physical aspects of fruits in which the concentration of phenols and ascorbic acid has positive relationship with fruit firmness, where losing firmness is a result of full maturity and fruits have lower ascorbic acid content (Blum et al., 2008). Oshiro et al. (2012) explain that the vitamin C content was better preserved in guava stored at 5°C and coated with 3% chitosan.

Texture

The texture of cagaita fruits was quantified by peak strain, which indicates the force required to break the cell walls of fruits. The peak strain is variable during storage (Figure 5). This parameter has not presented the desired values for any of the treatments, with decreases and increases during the storage period. Control fruits showed the best peak strain results, which varied less compared to the other treatments during the storage period. Fruits with 3% chitosan, which at the beginning of the storage period showed interesting texture values, confirmed more consistent bark and showed a significant decrease in texture values with increasing storage time. This significant decrease can be related to the ripening of fruits through a series of enzymatic reactions related to climacteric respiration and ethylene production (Castricini, 2009). The subsequent increase in peak strain is confirmed by the firmness of the cell wall due to the lack of degradation of insoluble protopectins to form soluble pectic acid and pectin in fruits (Maftoonazad and Ramaswamy, 2008). According to literature, the decrease in texture value can also be the result of hydrolases acting on the cell wall (Vicentini et al., 1999).

Fruit color

The brightness of cagaita fruits varied similarly for all treatments analyzed (Figure 6), showing an increase in the average L* value up to the tenth day of storage, and after, values decreased up to the end of the experiment. The L* parameter is related to brightness, which ranges from 0 (completely dark) to 100 (completely clear). With the application of chitosan, fruits had a matte appearance on the first day of storage, not as glossy as expected for cagaita fruits. With advancing storage, coated fruits were similar to uncoated fruits because chitosan remained with matte appearance. The cell walls of cagaita fruits showed symptoms of dryness, detached films with brittle appearance and depressions in the pulp, indicating that the coating film did not properly adhere to the cell wall of fruits. The matte appearance contributed to the decrease in fruit brightness values. This drop can also indicate that cagaita fruits undergone maturation processes and degradation reactions may have occurred, which contributed to the darkening of the fruit surface. Reis et al. (2006) reported that Japanese cucumbers also showed a slight increase in brightness and, later, values decreased up to the end of the analysis. The a* parameter varied similarly for control fruits and for those added with 3% chitosan; T3 fruits showed the lowest

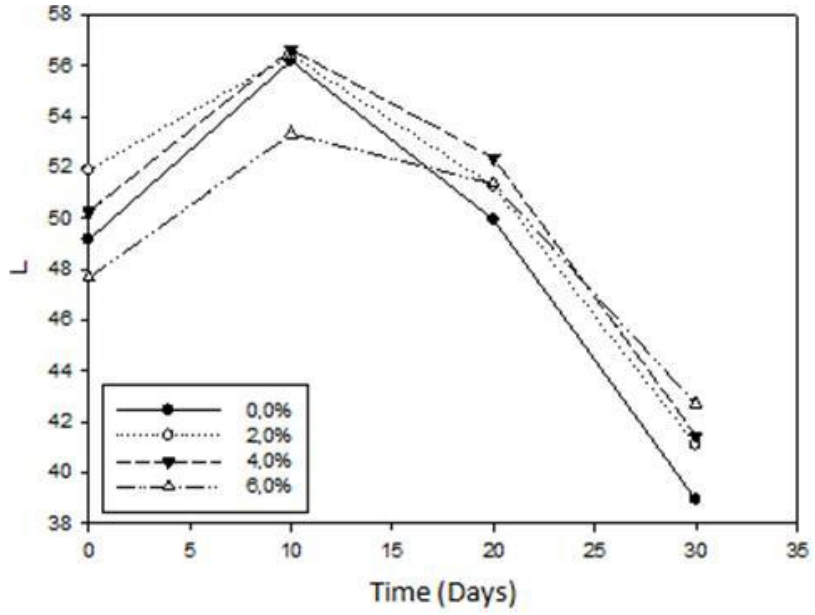

Figure 6 - L* parameter for control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

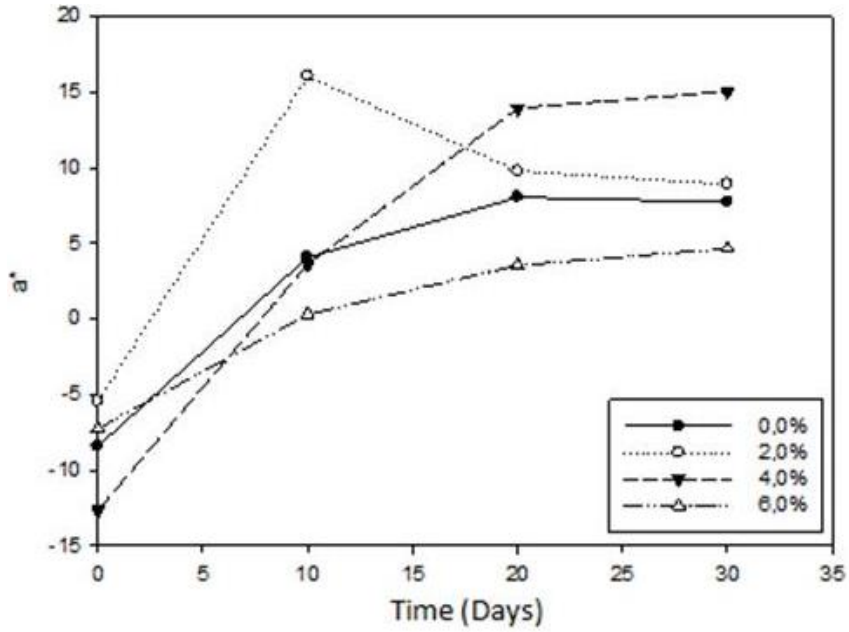

Figure 7. a* parameter of control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

mean values (Figure 7). For T2, variation is greater than that reported for T0 and T3 and for T1; variation did not follow the same behavior of other fruits analyzed. For low a * values, even negative, samples showed a more greenish coloration and for high a * values, samples showed red-purple color. The increased red values may be the result of maturation. According to Chitarra and Chitarra (2005), the color of fruits is related to the

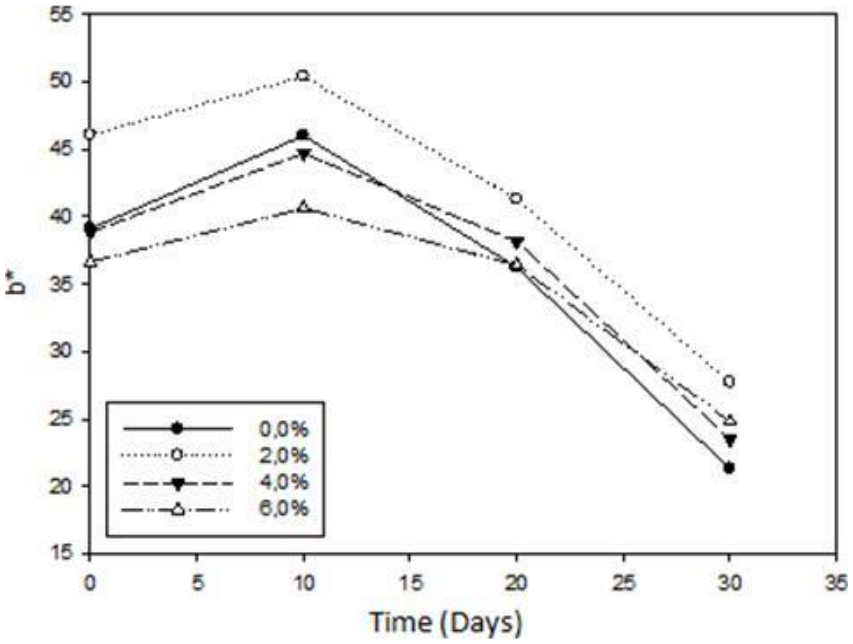

Figure 8. Color parameter b* of control fruits (T0) and treatments with 1% (v/v) (T1), 2% (v/v) (T2) and 3% (v/v) (T3) chitosan during storage of cagaita fruits under BOD at 12°C ± 0.1.

uniformity of the maturation stage. Santos et al. (2008) assessed the quality of peach cv. *Douradão* treated with 1% chitosan solution and found that the values of red coloration intensity decreased in peaches treated with chitosan, similarly to cagaita fruits coated with 3% chitosan in this study. Color parameter b * showed similar variation for all treatments, with greater variation for cagaita fruits coated with 1% chitosan (Figure 8). After the experiment, control fruits showed the lowest b * values. This parameter varies so that when low, including negative values, the sample shows blue color and when high, the sample is yellowish. Cagaita fruits showed no incidence of blue color on the outer surface and had a mixed green and yellow color. The decrease in yellow color during storage indicates that, correlating the changes in the green color, fruits underwent chlorophyll degradation and synthesis of yellow and red pigments that can be biosynthesized carotenoids (Vianna-Silva et al., 2008). The synthesis of these compounds is not interesting, as they indicate that cagaita fruits matured during the analysis, even coated fruits, and fruits coated with 1% chitosan showed the lowest incidence.

Scanning electron microscopy

The physical structure of cagaita fruits in the control treatment indicates the presence of heterogeneous surface (Figure 9), with formation of small bubbles and crusts indicated by arrows at the top and bottom of the image. The cell wall of the fruit does not show smooth appearance and can determine sites of gas exchange, water loss and entry of microorganisms that cause a drop in post-harvest quality and shelf life (Wu, 2010). Cagaita fruits coated with 1% chitosan (Figure 10) showed heterogeneous surface with incidence of pores. The presence of pores, especially in the amount indicated in the lower image shows that the cell walls of fruits can be more prone to gas exchange and water loss, which will result in loss of fruit quality, and such fact may occur with control fruits. Control fruits and those with 1% chitosan in the cell wall showed cracks on the physical structure. Such cracking may be due to the fact that wax production is disconnected from fruit growth, causing an imbalance of these parameters that may cause the formation of an interconnected network of channels on the fruit surface (Roy et al., 1994). The physical structure of cagaita fruits with 2% chitosan (Figure 11) indicates that the fruit surface was covered by a coating layer that has caused the appearance of higher points in relation to the remainder of the cover, indicated by arrows on the upper images. These points can be the result of misapplication of chitosan solution or poor adhesion to the cell wall of fruits. However, the lower image shows that there is formation of a network by the film solution, which may indicate that the solution adhered to the cell wall of fruits and when applied under the ideal conditions can contribute to maintaining product quality. The surface of cagaita fruits coated with 3% chitosan was more homogeneous compared to other physical structures

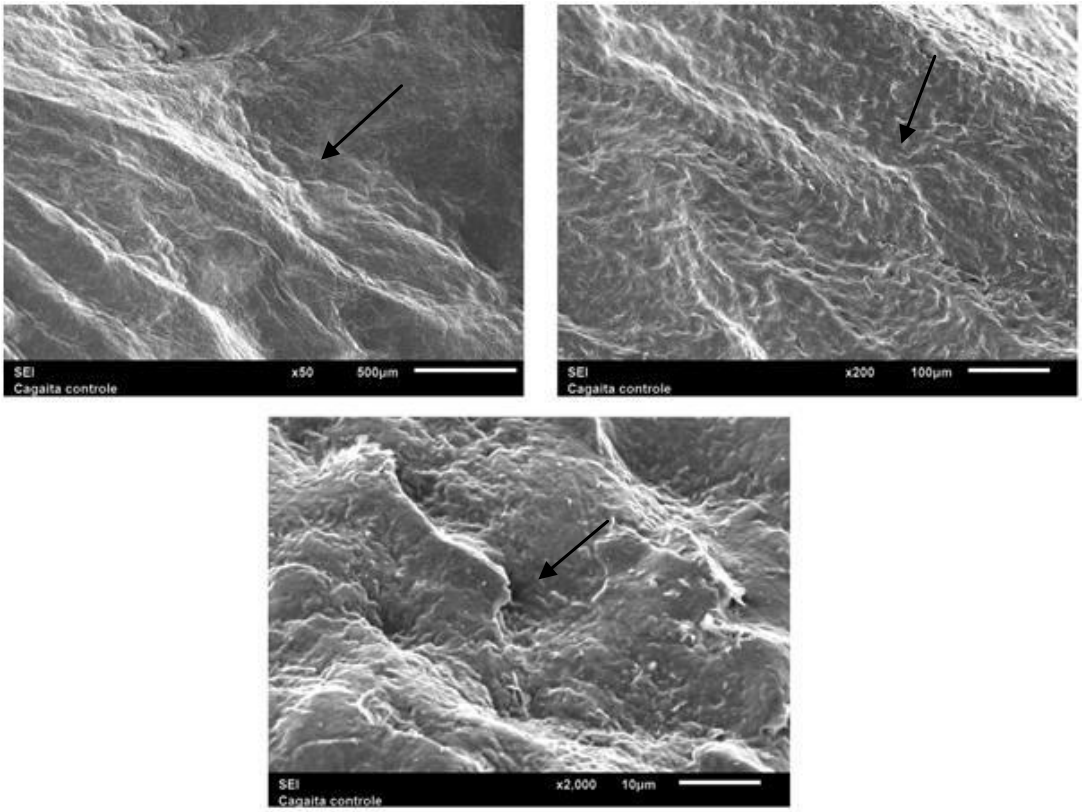

Figure 9. Physical structure (SEM) of control cagaita fruits (*Eugenia dysenterica* DC.). Fruit epicarp surface images.

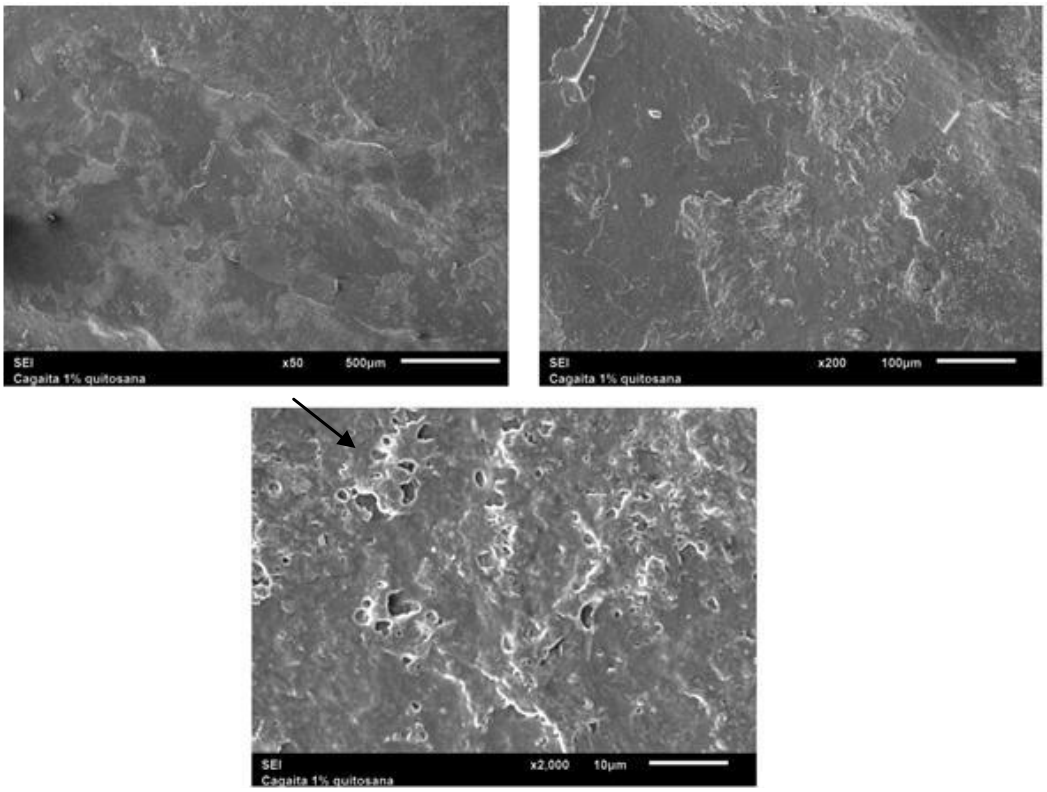

Figure 10. Physical structure (SEM) of cagaita fruits (*Eugenia dysenterica* DC.) coated with 1% chitosan. Fruit epicarp surface images.

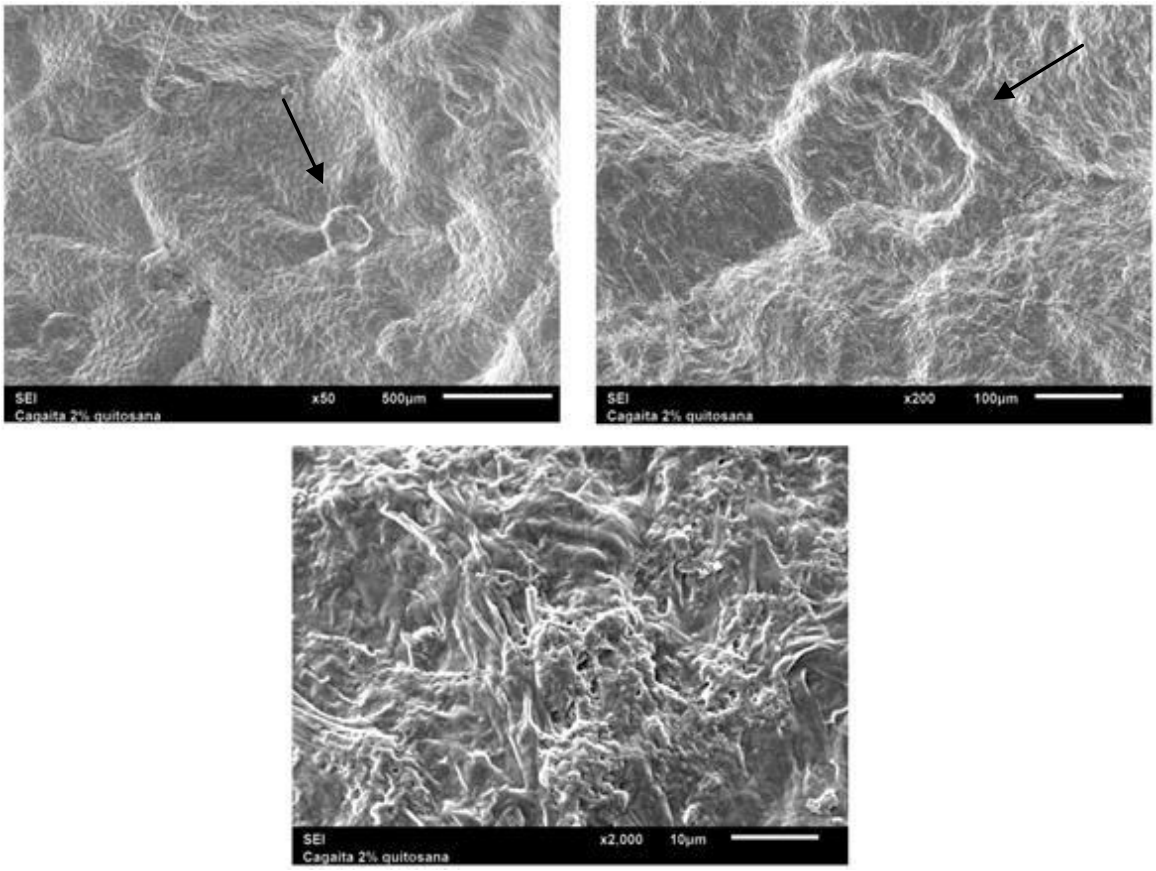

Figure 11. Physical structure (SEM) of cagaita fruits (*Eugenia dysenterica* DC.) coated with 2% chitosan. Fruit epicarp surface images.

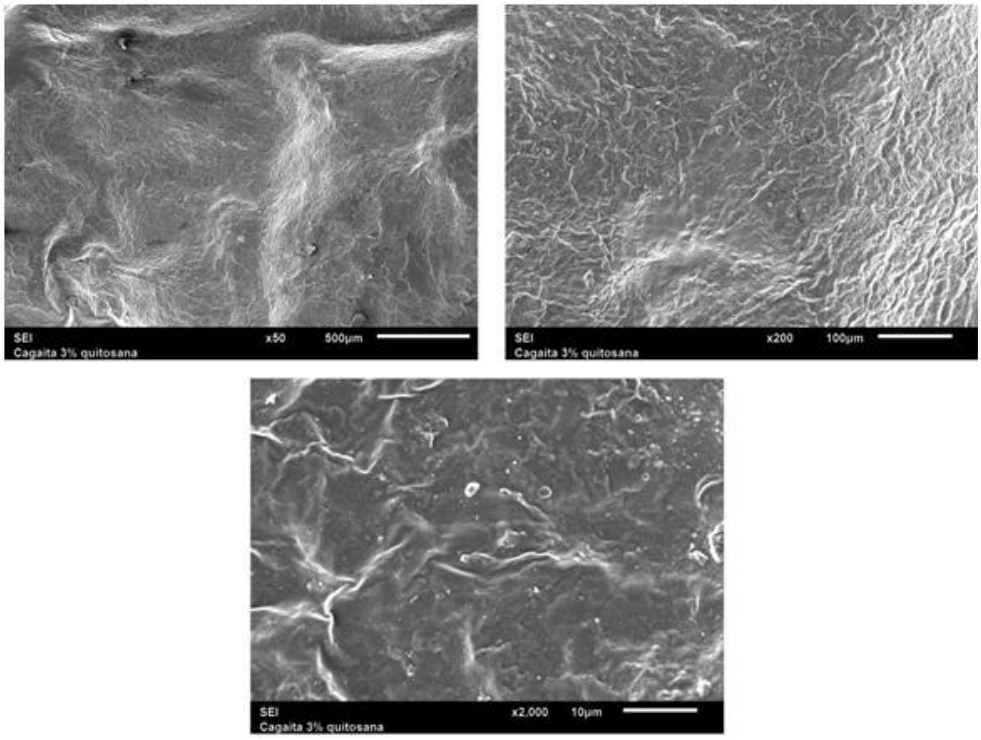

Figure 12. Physical structure (SEM) of cagaita fruits (*Eugenia dysenterica* DC.) coated with 3% chitosan. Fruit epicarp surface images.

analyzed in this work (Figure 12). Despite the presence of heterogeneous points, there is no incidence of bubbles or pores on the cell wall, which may indicate that such treatment is the most effective in maintaining fruit quality and increasing shelf life. The analysis of the physical structure of fruits through scanning electron microscopy contributes to evaluate the morphology of films due to detailed images of the cell wall surface (Freire et al., 2009). The drying process of the film and the nature of the hydrocolloid that change the interaction of components such as polysaccharides, plasticizers and water was also analyzed (Meneguim, 2012).

Conclusion

Chitosan solutions did not show the expected result in the conservation of cagaita fruits, and all parameters had values close to those analyzed in control fruits. However, this occurrence may be related to the presence of pores and poor adhesion of the solution to the cell wall of fruits as demonstrated in the physical structure analysis. Fruits that showed the best results, along with control fruits, were those coated with 3% chitosan, which suggests that further studies with higher chitosan concentrations could obtain more desired results for the conservation of fruit quality.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Effects of Nigeria Ekete light crude oil on plasma electrolytes, packed cell volume (PCV) and lipids profile in wistar *(Rattus norvegicus)* rats

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Hydrocarbon is known to alter blood parameters; some of these blood parameters may affect the activities of certain systems like cardiovascular system. Hydrocarbon gets into man and animal either through ingestion of contaminated food and water, bio-concentration through food chain, occupational exposure or by using hydrocarbon products. Effects of crude oil on plasma electrolytes, packed cell volume (PCV) and plasma lipid profile were examined in male *Rattus norvegicus* in this study. Crude oil was administered orally at dosages of 5 ml/kg (group I), 10 ml/kg (group II) and 20 ml/kg (group III) for two weeks while the control group received drinking water only. The result shows a reduction in packed cell volume in all treated groups compared to control (37.14±0.85, $P < 0.05$) and a significant ($P < 0.05$) increase in plasma sodium level in treated groups compared to control (22.486±2.983 mg/L) while there was no significant alteration in plasma lipids profile of the treated groups relative to the control. Oral administration of crude oil significantly altered the plasma levels of electrolytes and PCV.

Key words: Electrolytes, crude oil, plasma lipids profile, packed cell volume.

INTRODUCTION

Crude oil, the corner stone of Nigeria's economy has been posing a threat through contamination of immediate environment during exploration and exploitation (Amadi et al., 1993). Hydrocarbons eventually get into man and animal through ingestion of contaminated food and water, bio-concentration through food chain, occupational exposure or by using hydrocarbon products (Amadi et al., 1993; Egborge, 1991). However, many people in some communities ingest crude oil directly as remedy for various conditions such as snake poisoning, convulsion, treatment of skin infection (Adesanya et al., 2009), gastrointestinal disturbances (Eyong et al., 2004) and arthritis. Crude oil has been shown to be well absorbed through all routes of contact such as dermal, oral or respiratory, because it is highly lipophilic (Bohlen et al., 1997). Impurities in crude oil include phenolic acid, napthelic acid and small quantities of most known elements except: sulphur, nitrogen, nickel, molybdenum which is present in relatively large quantities (Mason, 1966).

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Environmental pollutants are known to alter hematological parameters which in turn affect the functioning of vital organs like heart, blood vessels or the cardiovascular system as a whole by inducing; endothelial oxidative stress and vasodilator dysfunction (Podlutsky et al., 2010), dyslipidemia (Lemonine, 1911). Also increase in plasma electrolytes is known to contribute to incidence of hypertension

The ingestion of crude petroleum contaminated diet imposed a reciprocal relationship between high-density lipoprotein (HDL)- cholesterol and low-density lipoprotein (LDL)- cholesterol in the plasma of rabbit and reduced blood glucose (Achuba, 2005; Ben-David et al., 2001). Presence of *12·5–50·0* ppm dispersed crude oil in solution prevented the development of high mucosal transfer rates in the ducklings given hypertonic saline drinking water(Crocker et. al. 1974). Alkindi et al. (1996), showed that water soluble fraction of Omani crude oil resulted in a progressive increase in plasma cortisol concentrations from 3 h onwards (rising from 18 to 51 ng ml^{-1} after 48-h exposure), increased plasma glucose concentration, did not affect plasma osmolality, sodium and chloride concentration but caused decrease in plasma potassium concentration in Flounders *Pleuronectesflesus.*

Analysis of osmo-regulation of the resident estuarine fish *Atherinella brasiliensis* 1^{st} month, 4^{th} month, and 7^{th} month after oil spill in Paranaguá Bay, Brazil, showed an increase in plasma osmolality (reaching ~525 mOsm/kg H_2O, or ~70% above values in reference fish) and chloride (reaching 214 mM or ~51% above values in reference fish) were detected 4 months after the spill; Plasma cortisol concentration increased progressively in samples from fish obtained 4^{th} month (462 ng/ml) and 7^{th} month (564 to 650 ng/ml) after the spill, compared to values in reference fish (192 ng/ml),(Souza-Bastos and Freire, 2011). Alonso-Alvarez et al. (2007), reported that gulls (laridae) fed with prestige fuel oil showed reduced glucose, inorganic phosphorus levels in plasma, and significantly reduced creatinine values. Glucose concentration was inversely related to Total Polycyclic Aromatic Hydrocarbon (TPAH) levels. Males gulls fed with fuel oil showed higher plasma activity of aspartate amino transferase (AST) than controls, and plasma activity of gamma-glutamyl transferase (GGT) was reduced. Shakirov, (2001).

Crude oil contains chemicals that readily penetrate cell walls, damage DNA, and alter the functions of the cells of any organ in the body (CDC, 1999; Achuba and Osakwe, 2004; EPA, 2010). The aims of this study were to examine the effects of oral administration of crude oil on plasma electrolytes, packed cell volume and plasma lipid profile in male *R. norvegicus*.

MATERIALS AND METHODS

Twenty eight male *R. norvegicus* of average weight of 150 g bought from animal farm from Ibadan were used for this experiment. They were housed in rat cages and acclimatized for two weeks before the commencement of the experiment. They were fed with commercially prepared rat diet and allowed access to water *ad libitum* throughout the period of the study. The rats were randomly divided to four groups (n=7). Crude oil was not administered to the control group but was given water as placebo. Rats in test received Nigerian Ekete crude oil orally every day for two weeks: 5, 10 and 20 ml/kg respectively were given per rat in group I, group II and group III. Body weights were measured at the end of two weeks before collecting blood sample through peri-orbital sinus. Samples were collected into lithium heparinised sample bottles. They were centrifuged for lipid profile and electrolyte analysis. EDTA sample bottles were used to collect samples for packed cell volume analysis.

Electrolytes analysis

The electrolytes were analyzed for plasma calcium, potassium, and sodium concentration using Atomic absorption Spectrophotometer (Walsh, 1955).

Digestion of the sample

10 ml of Aqua radial (H_2SO_4/ HCL ratio 1:3) (H_2SO_4) was added to the sample inside 250 ml conical flask, the mixture was evaporated inside fume cupboard until brown fume disappear leaving white fumes. Then it was distilled and water was added up to 50 ml mark and filtered into sample bottle for analysis.

Standard preparation

The standard of calcium, sodium and potassium were prepared by dividing the molar mass of the compound of the elements by the molar mass of the elements, the product of the division is dissolved in 1litre of 5% nitric oxide, and this is stock standard (1000ppm). Serial dilution was used to calibrate the instrument. C1V1=C2V2.

PCV analysis

Packed cell volume was determined by micro-heamatocrit centrifugation (Jain, 1986)

Lipid profile analysis

Total cholesterol, HDL-cholesterol and triglyceride was determined through spectrophotometric method using , BIOLABO SA CHOD-PAP reagents (Ref 80106), BIOLABO S-HDL-CHOLESTEROL (PTA) and BIOLAB reagents – GPO respectively while LDL-cholesterol was calculated using this formular; (using the fried Waldes formula)

LDL cholestrol = Total cholesterol – TG / 2.2 + HDL-cholesterol (mmol/L)

The analysis of variance (one way ANOVA) test was carried out using SPSS 15.0 to analyse the level of significant between the control group and treated groups. The level of significance for the ANOVA test was taken at $P < 0.05$.

RESULTS

Plasma electrolytes showed inconsistent variation in treated groups. There was significant increase in plasma Na^+ in treated

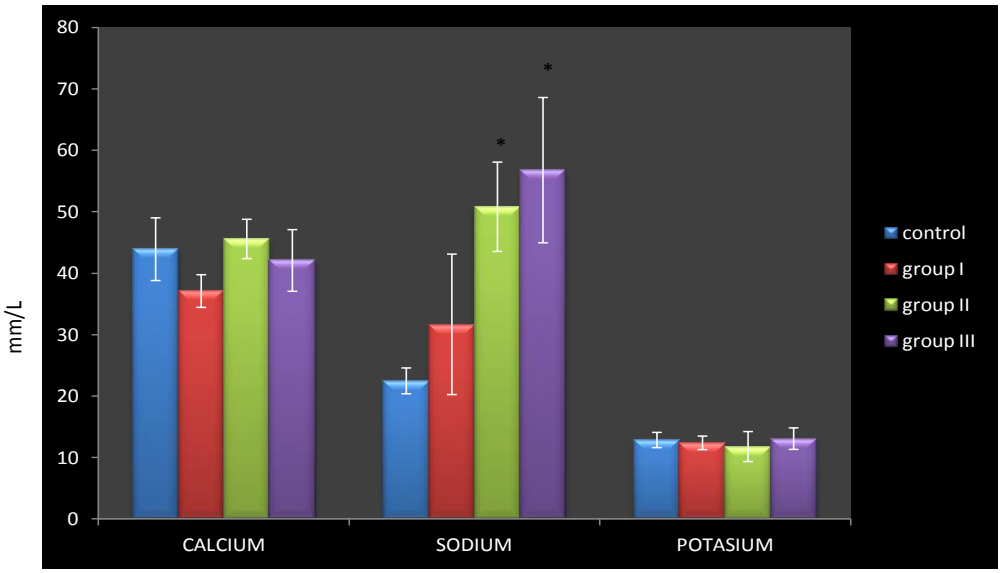

Figure 1. Changes in plasma electrolytes due to oral administration of crude oil. Asterike (*) indicates significant difference that is P-value is <0.05.

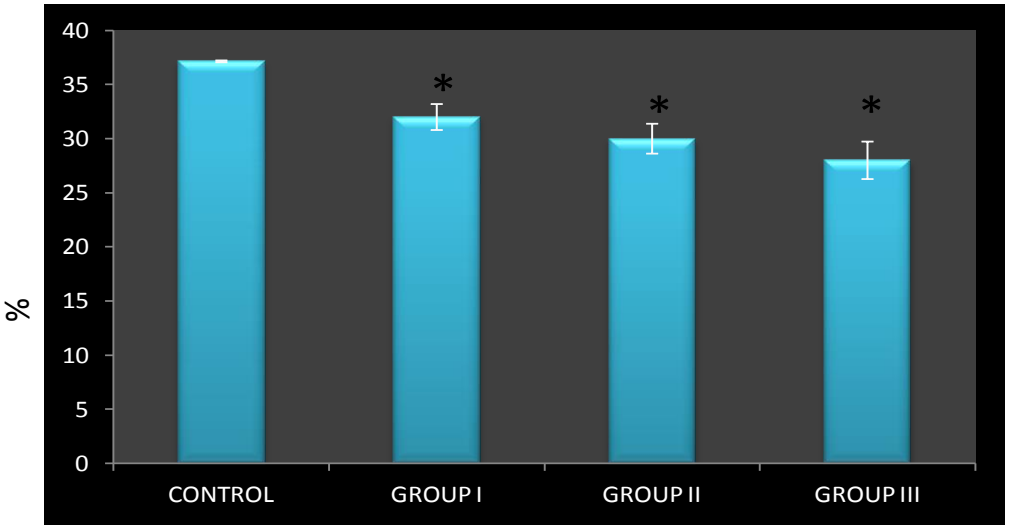

Figure 2. Effects of oral administration of crude oil on packed cell volume (*) indicates significant difference that is P-value is <0.05.

groups compared to control (22.486±2.983) ($P < 0.05$) while there was no significant difference in plasma K^+ and Ca^{2+} in treated groups compared to control as shown (Figure 1). The packed cell volume was significantly lower in all treated groups compared to control (37.143±0.857%) (Figure 2). Table 1 shows the effects of oral administration of crude oil on plasma lipid profile. The results show that there was no significant difference between the treated groups and control.

DISCUSSION

Exposure to hydrocarbon may vary from negligible persistent unconscious exposure to considerably large single doses (as in drinking as remedy). Effects of crude oil on plasma electrolytes, packed cell volume and plasma lipids profile were examined in this study. This study shows a consistent increase in plasma sodium level relative to the increase in dosage in treated groups while there was no significant difference in plasma level of calcium and potassium. Different authors have observed that crude oil alters plasma constituents, including plasma enzymes, electrolytes and amino acids. Findings in this study contradicted the observation of Alkindi et al. (1996), who showed that water soluble fraction of Omani crude oil did not affect plasma

Table 1. Effects of oral administration of crude oil on plasma lipid profile.

Parameter mmol/l	Control	Group I	Group II	Group III
Total cholesterol	2.5143±0.13171	2.1000±0.09258	2.3429±0.13067	2.7429±0.20800
HDL cholesterol	1.5571±0.10659	1.5143±0.11004	1.7143±0.14046	1.6286±0.13222
LDL cholesterol	0.5143±0.09932	0.3857±0.11429	0.4286±0.09932	0.6571±0.15253
Triglyceride	0.8571±0.09476	0.5714±0.04206	0.7214±0.05329	1.0143 ±0.24244

There was no significant difference between treated groups and control.

osmolality, sodium and chloride concentration but caused decrease in plasma potassium concentration in *Flounders Pleuronectesflesus.* Gad (2011), observed an initial decrease in serum potassium and sodium and subsequence increase in potassium and sodium in *oreochromis niloticus* exposed to crude oil pollution.

Increase in plasma osmolality (reaching ~525 mOsm/kg H_2O, or ~70% above values in reference fish) and chloride (reaching 214 mM or ~51% above values in reference fish) were detected 4 months after crude oil spill in Paranaguá Bay, Brazil by Souza-Bastos and Freire, (2011). Alonso-Alvarez et al. (2007), fed gulls with Prestige oil; they reported that gulls fed with fuel oil showed reduction in glucose and inorganic phosphorus levels in plasma, as well as a trend to significantly reduced creatinine values. Shakirov (2001), reported decreases in the levels of K, Mg, and Ca and increases in the concentrations of Na and P in the red blood cells and in those of K, Mg, and P in the plasma while plasma Na and Ca were reduced in workers exposed constantly to hydrocarbon inpetroleum-refining industry for 3 to 5 years. Alteration in plasma electrolytes level is related to the development of hypertension (Moore, 1989).

The Packed cell volume (PCV) gave indication on haematopoietic activity taking place in the bone marrow. This study revealed significant reduction in packed cell volume of the treated groups, thus corroborating the previous findings on the effects of hydrocarbon on erythropoietin. It has been shown that hydrocarbon causes bone marrow hypoplasia, consequently reducing red blood cell and white blood cell formation (Eyong et al, 2004, Cody et al., 1981). Leighton (1990), reported heamolytic anaemia in mice given crude oil. Cody et al. (1981), reported heamatological disorder evidenced by a decrease in PCV and red blood count in rat exposed to 1, 3 dinitrobenzene. Hydrocarbon has been reported to contribute to hemolytic anemia by Eyong et al. (2004).

Though several authors have shown that crude oil ingestion causes abnormality in lipid profile, the results of this study shows that oral administration of crude oil did not alter lipid profile significantly. Achuba (2005) discovered reciprocal relationship between HDL-cholesterol and LDL-cholesterol in the plasma of rabbit fed with crude petroleum contaminated diet. Anigbogu and Ojo (2009), observed alteration in lipid profile of rats given different fractions of petroleum products. Epidemiological study has also shown that elevated concentrations of total cholesterol and LDL-cholesterol in the blood are powerful factor for coronary heart disease (Lawn, 1999). Dyslipidemia is known to be co-risk factor for development of hypertension along with obesity and diabetes, by causing endothelia dysfunction; a dysfunctional endothelium will express impaired nitric oxide production activity as well as alteration in endothelin I and endothelin A and B receptors expression (Ruben et al., 2006).

Conflict of interests

The authors did not declare any conflict of interest.

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African Journal of Biotechnology

Full Length Research Paper

Therapeutic role of glucogalactan polysaccharide extracted from *Agaricus bisporus* on trimethyltin chloride induced neuropathy in rats

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Trimethyltin (TMT) chloride induces limbic system neuro-degeneration, resulting in behavioral alterations like cognitive deficits. This study investigates the effect of glucogalactan polysaccharide (GA) extract, which was purified from the roots of *Agaricus bisporus* mushroom, on trimethyltin chloride (TMT) induced neuropathy in rats. Adult male rats (200 ±10 g) were divided into four groups that were fed with basal diet throughout the experiment (28 days). The first group (G I) was control group, the second group (GII) was treated with 300 mg/kg BW GA intraperitoneally (i.p.) daily for 28 day. The third group (GIII) was administered i.p. with 8.0 mg TMT /kg body weight (BW), and the fourth (G IV) was treated like the third group and was injected with GA 300 mg/kg BW daily for 28 day after 48 h of TMT. Many bioactive compounds, which were found in GA did not cause any changes in the second group compared to normal control group. The results reveal that GA given 48 h after TMT treatment has excellent neuropathy effect, lowers the average of MDA, HSP70, homocystein and the neurotransmitters in brain tissue homogenate; they were markedly reduced by the administration of GA to almost normal levels. Neurotransmitters and nitric oxide were significantly increased in the group given GA treatment compared to TMT group. The comet assay for DNA revealed that, TMT induced statistically significant ($P<0.05$) increase in the mean value of the tail length and tail moment of the rats. They significantly decreased after GA treatment, suggesting alleviated oxidative stress mediated by TMT. GA administered TMT-treated rats had improved brain histology, diminished level of MDA and nitric oxide (NO) in brain tissue and enhanced total antioxidant capacity in serum compared to TMT group. It could be concluded that GA plays a positive role in the improvement of brain function after TMT-induced neuropathy. Taken together, our results suggest that GA will be useful in developing strategies for protecting nervous system and improving the brain.

Key words: Trimethyltin, neuro-degeneration, *Agaricusbisporus*, glucogalactan.

INTRODUCTION

Mushrooms comprise a vast and yet largely untapped source of powerful new pharmaceutical products. Nowadays, biochemical and nutritional researchers pay more attention to mushroom polysaccharide due to its various biological functions in food, health care or medicine; it has antioxidant, immunostimulatory and antitumor effects (Li et al., 2009). Basidiomycetes present different kinds of glucans and heteropolysaccharides. The common monosaccharide composition of these polysaccharides is glucose, galactose, xylose, mannose

and fucose. Generally, (1→3), (1→6)-β-glucans are extracted from these mushrooms, and also galacto-mannans, heteroglycans, and fucogalactans (Moradali et al., 2007). Mushrooms included in the same genera show more similarities in their composition, including the structure of carbohydrates (Zhang et al., 2007). Mushroom polysaccharides have traditionally been used for the prevention and treatment of a multitude of disorders like infectious illnesses, cancers and various autoimmune diseases. Bioactive polysaccharides are recognized by membrane receptors in leukocytes and macrophages, leading to proliferation and differentiation of immune cells (Moradali et al., 2007). These activities are responsible for enhancing the innate and cell-mediated immune responses, and consequently, for the induction of antitumoral and bactericidal effect (Lull et al., 2005). Besides the well-known antitumor β-(1→3)-glucans, a wide range of biologically active glucans with other structures have been described. These polysaccharides have linear or branched molecules in a backbone composed of α- or β-linked glucose units, and they contain side chains that are attached in different ways. Heteroglucan side chains contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose as a main component or in different combinations. Glycans, in general, are polysaccharides containing units other than glucose in their backbone. They are classified as galactans, fucans, xylans and mannans by the individual sugar components in the backbone. Heteroglycan side chains contain arabinose, mannose, fucos, galactose, xylose, glucuronic acid and glucose as a main component or in different combinations. Mushroom polysaccharides exert their antitumor action mostly via activation of the immune response of the host organism. These substances are regarded as biological response modifiers (Wasser and Weis, 1999). This basically means that: (1) they cause no harm and place no additional stress on the body; (2) they help the body to adapt to various environmental and biological stresses; and (3) they exert a nonspecific action on the body, supporting some or all of the major systems, including nervous, hormonal, and immune systems, as well as regulatory functions (Brekhman, 1980).

The number of mushrooms with known pharmaco-logical qualities is much lower still. Isolation and purification of polysaccharides from mushroom material is relatively simple and straightforward, and can be carried out with minimal effort. Mycelia formed by growing pure cultures in submerged conditions are of constant composition, and submerged culture is the best technique for obtaining consistent and safe mushroom products (Borchers et al., 1999; Wasser et al., 2000). Therefore, discovering novel structurally and biologically poly-saccharides from mushroom, especially those unexploited species has become a hot spot of great interest. The present study was carried out in an attempt to investigate the neuro-protective effect of GA polysaccharide from *Agaricus bioporus* in rats.

MATERIALS AND METHODS

Laboratory animals and experimental design

Dawley rats (weighing 180 ± 20 g) were purchased from the laboratory animal colony at the Institute of Ophthalmology, Cairo University, Egypt. All rats were provided with food and water *ad libitum* throughout the experimental period. The experiment was carried out in accordance with the guidelines of the experimental animal ethics. After seven days of acclimatization, rats were randomly assigned into four groups (eight per group); the first group (GI) acting as a control received saline (0.5 ml) for 28 days. The second group (GII) received GA (300 mg/kg) intraperitoneally (i.p.). The third group (G III) rats were injected intraperitoneally (i.p.) with TMT (8.0 mg/kg, body weight) dissolved in 0.9% saline and then received saline after 48 h of TMT for 28 days. The fourth group (GIV) was administered GA (300 mg/kg, i.p.) like the second group after 48 h of TMT. Anesthetic procedures and handling with animals were approved by and complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt (Approval number 10082).

Chemicals

Trimethyltin chloride (TMT) was purchased from Sigma-Aldrich Co. (St. Louis MO), and solvents were from E. Merck (Darmstadt, Germany).

Extraction and purification of the polysaccharide

Fresh fruit bodies (1 kg) of *A.bisporus* were collected from Bloshia Mushroom Company in Dokki, Egypt (Mahmoud et al., 2014). The sample was washed with distilled water and boiled in a water bath for 6 h. The mixture was kept overnight at 4°C and filtered through fresh linen cloth. The filtrate was centrifuged at 5000 rpm (Sigma-Laborzentrifugen, 2K 215, Sigma Co., and D37520 Osterode-am-Harz, Germany) for 45 min at 4°C. The supernatant solution was collected and the polysaccharide (CAB) was precipitated with EtOH

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Abbreviations: CA1, Cornu ammonis 1; **CA3,** cornu ammonis 3; **AB,** water soluble; **ABI,** water insoluble; **CAB,** crude of aqua's polysaccharide; **CSF,** cerebrospinal fluid; **AchE,** acetylcholine esterase; **DA,** dopamine; **Da,**dalton; **EtOH,** ethanol; EPS: exopolysaccharide; **5-HT,** serotonin; **MDA,** malondialdehyde; **NO,** nitric oxide; **IL-1,** interleukin 1; **IL-3,** interleukin 3, **NAOH,** sodium hydroxide; **GMA,** glucogalactan from mushroom (*Agaricus bisporus*); **FTIR,** Fourier transforms infrared; **KBr,** potassium bromide; **i.p,** intraperitoneal.

(1:5, v/v). After keeping the precipitated material in the mixture overnight at 4°C, it was centrifuged at 4°C for 1 h, and then the residue was freeze-dried (1.5 g). The dried material was dissolved in 4% NaOH solution and re-precipitated with ethanol. The re-precipitated material was collected throughcentrifugation and dissolved in minimum volume of water. The solution was then dialyzed against distilled water for 30 h (3x 1000 mL) to remove alkali and low molecular weight materials. During this dialysis, one portion got precipitated from this solution. The whole dialyzed solution was centrifuged at 8000 rpm and 4°C. The water soluble (AB) and insoluble (ABI) parts were lyophilized separately (Hana et al., 2010).

Isolation and purification of exopolysaccharide

Inoculum was prepared by transferring one loop full of culture from *A. bisporus* slant to an Erlenmeyer flask (250 mL) containing 50 mL seed medium containing (g/L) sucrose, 20; yeast extract, 2; peptone and 75% sea water (Jiang et al., 1999). The seed cultures were grown at 37°C on a rotary shaker incubator at 150 rpm for 24 h. After incubation, 3 mL of the seed culture was transferred into an Erlenmeyer flask (250 mL) containing 50 mL of fermentation medium (g/L) sucrose, 50; peptone, 4; yeast extract, 2 in 75% sea water pH 7.0 (Read and Costerton, 1987). The fermentation cultures were then incubated at 37°C with shaking at 150 rpm for three days. The exopolysaccharide (EPS) sample was prepared from *A. bisporus* culture in the fermentation medium. The fermented broth was collected and centrifuged at 5000 rpm and 4°C for 20 min. It was dialyzed three times (1000 mL × 3) under a flowing tap-water, in a dialysis tube (MWCO 2000) for 24 h. The dialyzed solution was precipitated with four volume chilled ethanol; the precipitate was washed with acetone, diethyl ether and dried at 50°C until constant weight. The crude EPS was re-dissolved in deionized water and forced through a filter (0.45 mm); it was then applied to a column (2.5 × 70 cm, i.d.) of DEAE-cellulose. After loading with sample, the column was eluted with gradient NaCl solution (0.0 to 1.0 M), and the procedure was monitored by the phenol-sulfuric acid method mentioned above. One polysaccharide active fraction (GA) was collected, dialyzed and lyophilized. GA was used for activity assessment and structural analysis (Asker et al., 2009). Nevertheless, other macromolecules, such as proteins, may be also present in the medium. Therefore, several purification steps must be carried out to remove other substances. The samples were subjected to methanolic extraction in order to remove phenolic compounds, monosaccharides, amino acids and other related molecules. The elimination of phenolic derivatives was successful (Palacios et al., 2011), and it increased the effectiveness of the extraction (Park et al., 2009). Proteins were removed by precipitating with trifluoroacetic acid (20%, w/v) or treating with enzyme protease at 40°C for 1 h (pH 7.5). The proteins were separated by centrifugation. After protein removal, polysaccharides were precipitated from the supernatants by the addition of ethanol in 2:1 ratio (v/v). Concentrated sodium chloride solutions were added to favor the precipitation, and the solid was washed with organic solvents, such as acetone or ethanol. Once the polysaccharides were separated from other compounds, pure carbohydrate fractions were obtained; however, each fraction contains several polysaccharides showing different molecular sizes. Fractionation was performed by precipitating with ammonium sulfate.

Chemical analyses

The purified GA (50 mg) was subjected to hydrolysis with 6N HCl for 4 h at 100°C in a sealed tube. Excess acid was removed by evaporation in a water bath at a temperature of 40°C and co-distilled with water (1 mL × 3) (Sudhamani et al., 2004). Uronic acid contents were determined by measuring the absorbance at 525 nm using the m-hydroxybiphenyl colorimetric procedure and with glucuronic acid as the standard (Filisetti-CozziandCarpita, 1991). Sulfate was measured using the turbidimetric method (Dodgson and Price, 1962) with sodium sulfate as standard. N-acetyl glucose amine was estimated by the Morgan and Elson reaction (1934). UV–vis spectroscopy analyses were conducted on ultraviolet–visible–near-infrared spectrophotometer (2401PC (Shimadzu, Japan). The polysaccharide solution was prepared by suspending the sample in distilled water to a concentration of 1.0 mg/mL for UV–vis measurement in the wavelength range of 190 to 700 nm. Monosaccharide compositions of the GA were determined by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9 mm × 30 cm, i.d.), using deionized water as the mobile phase (flow rate 0.5 mL/min), as described by El-Sayed et al. (2005).

Molecular weight determination

The molecular weight of the polysaccharide was determined by gel permeation chromatography (GPC) on Agilent 1100 series, Germany, Detector: Refractive Index FPl gel particle size (5µm), 3 columns of pore type (100, 104 and 105 A°) on series, length 7.5 × 300 mm (1000, 5000000 Da) for DMF solvent Styrogel HR-DMF, 3 µm (7.8 × 300 mm), Water Company Ireland; one column (5000 to 600000 Da) for water solvent (polyethylene oxide/glycol standard) PL aqua-gel-OH 7.5 mm and 30 µm pore type; 8 µm particle size; PL aqua-gel-OH 7.5 mm, 50 µm pore type and 8 µm particle size, in series Mw from 100 to 1250000 g/mol. The sample (0.01 g) was dissolved in 2 ml of solvent, and then filtrated by siring filter (0.45). Then the sample was put in GPC device. The polydispersity index was calculated from the Mw/Mn ratio (You et al., 2013).

Infrared spectroscopy

The Fourier-transform infrared (FT-IR) spectrum of the polysaccharide was measured using a Bucker scientific 500-IR Spectrophotometer. The exopolysaccharide was mixed with KBr powder, ground and pressed into 1 mm pellets for FTIR measurements in the range of 400 to 4000 cm^{-1} (Ray, 2006).

***In-vitro* antioxidant studies**

Free radical scavenging effect

The free radical scavenging activity of AB and standard compounds at different concentrations (75, 150, 300 and 600µg/ml) was measured by 1,1-diphenyl-2-picrylhydrazil (DPPH) using the method of Yamaguchi et al. (1998). Briefly, 0.1 mM solution of DPPH in ethanol was prepared. Then, 1 ml of this solution was added to 3 ml of samples and standard solution Vitamin C (VC) and Butylated hydroxytoluene (BHT) at various concentrations of the polysaccharide. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, the absorbance was measured at 517 nm in a spectrophotometer (Schimadzu UV/VIS-240IPC). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH radical concentration in the reaction medium was calculated from the following equation: DPPH scavenging effect (%) = 100 − [(A0-A1)/A0) x100], Where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the sample of polysaccharide (Oktay et al., 2003).

***In-vivo* study**

Collection of blood and tissue samples

At the end of the 28th day of the trial, blood samples were collected

from all groups by cardiac puncture into clean dry centrifuge tubes. Subsequently, the brain tissues of the animals were excised. The blood samples collected were centrifuged for 15 min at 3000 rpm to separate serum. Serum was carefully aspirated and transferred into dry clean Wasserman and kept frozen at -20°C until analysis. The brain tissues were washed with deionized water for the removal of blood. Homogenization was performed in a phosphate buffer solution with pH value adjusted to 7.4, and later the supernatant was separated by means of centrifugation at 20.000 rpm for 1 h. The supernatant and hemolysate obtained were used for the analyses of certain parameters.

Biochemical analysis

Lipid peroxide concentration was determined by measuring brain-malondialdehyde (MDA) according to the method of Yoshioka et al. (1979).Nitric oxide was determined according to Miranda et al. (2001). The acetylcholine esterase (AchE) was measured by the method of Ellman et al. (1961), which involves the formation of a yellow colour due to the reaction of thiocholine with dithiobisnitro-benzoate ions. Determination of homocysteine was carried out using HPLC (Varian Inc., CA, USA) attached to fluorescent HPLC detector according to the method of Dimitrova et al. (2001). Noradrenaline (NA) was measured using appropriate [125I] radioimmunoassay kit (IBL, Hamburg, Germany) while dopamine (DA) and seratonin (5 HT) were estimated in the brain tissues of rats according to Zagrodzka et al.(2000). Serum total antioxidant capacity was estimated in serum by colorimetric method according to the method of Koracevic et al. (2001). Total heat shock protein 70 (HSP70) levels were determined by ELISA technique (Life Science Inc.).

Detection of oxidative DNA damage (comet assay)

To investigate the *in vivo* genotoxicity effect of the TMT, a single-cell gel electrophoresis analysis (comet assay) was used, which is a simple, rapid, and sensitive technique for detecting DNA damage at the level of individual eukaryotic cells (Singh et al., 1988; Zegura and Filipic, 2004). Crushed samples of 2 gm were transferred to 1 ml of ice-cold PBS. This suspension was stirred for 5 min and filtered. The cell suspension was mixed with an equal volume of 1% low-melting-point agarose at 37°C and quickly pipetted onto the first agarose layer in the same manner. Finally, 70ml of 0.5% low-melting-point agarose was added to cover the cell layer. The slides sandwiched without coverslips were immersed in freshly prepared cold lysing buffer [2.5 mol/l NaCl, 100 mmol/l Na2-EDTA, 10 mol/l Tris, 1%N-lauroyl sarcosine sodium salt (pH 10) with 1% Triton X-100 added just before use] and kept at 4°C for 45 min to 1 h. The slides were placed on a horizontal gel electrophoresis platform and covered with cold alkaline buffer (300 mmol/l NaOH and 1 mmol/l Na2-EDTA) for 8 to 20 min in the dark at 4°C to allow DNA unwinding and expression of the alkali-labile sites. Electrophoresis was conducted at 41°Cin the dark for 20 min at 25 V and 300 mA. The slides were then rinsed gently twice with neutralizing buffer (0.4 mol/l Tris, pH 7.5). Each slide was stained with 50mlof ethidium bromide at a concentration of 2 mg/ml and covered with a coverslip. They were then stored at 4°C in sealed boxes until analysis. A total of 100 randomly captured comets from each slide were examined at X 400 magnification using a fluorescence microscope connected to a CCD camera in an image analysis system [Comet 5 image analysis software developed by Kinetic Imaging Ltd. (Liverpool, UK)]. A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. To quantify the DNA damage tail length (TL), the percentage of migrated DNA (tail DNA %) and tail moment (TM) were evaluated. TL (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the center of the cell. Finally, the program calculates TM.

Histological examination

Autopsy samples were taken from the brain of rats in different groups and fixed in 10% formol saline for 24 h. Washing was done in tap water, then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slidge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylinand eosin stain for routine examination through light electric microscope (Banchroft et al., 1996).

Statistical analysis

The results were expressed as mean ± SD. The differences among means were analyzed through one way analysis of variance (ANOVA) followed by Duncan's post hoc analysis and the values ≤ 0.05 were considered significant. SAS software version 9.0 was used for the statistical analysis.

RESULTS

Analysis of monosaccharide composition of *A. bisporus*

The semi-purified polysaccharide extract was analyzed for monosaccharide composition. The monosaccharide composition was analyzed by HPLC. The main mono-saccharide of the AB and AB-I are glucose and galactose in a molar ratio of 6:1, respectively.

Structural characterization of GA extract

GABP extract from *A. bisporus* was confirmed by IR as shown in Figure 1. The bands in the region of 3463.53 cm^{-1} were due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2927.41 cm^{-1} were due to C–H stretching vibration, and the bands in the region of 1664.27 cm^{-1} were due to associated water. The characteristic absorptions at 833.09 cm^{-1} in the IR spectra indicated that α- configurations were simultaneously present in *A. bisporus*. The actual molecular weight and distribution of the AB were determined by gel permeation chromatography (GPC). The polysaccharide in the GPC chromatogram (Figure 1) was widely dispersed molecules polydispersity index of 1.79 and had an overall weight average molecular weight (Mw) of 2.08 × 106 g/mol and number average molecular weight (Mn) of 1.61×10^6 g/mol.

Effect of GA on lipid peroxidation

The results of the present study showed significant

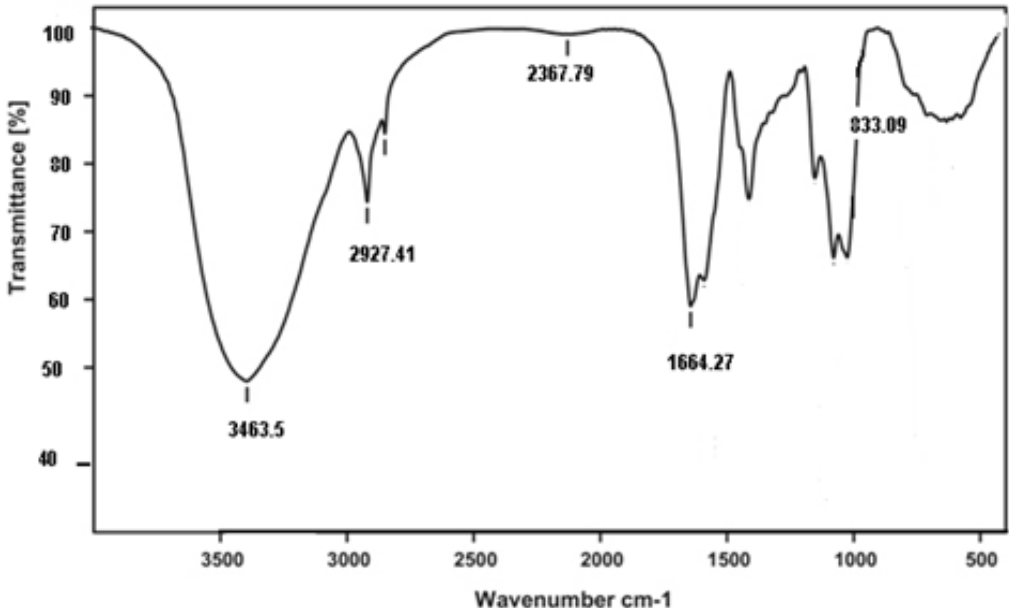

Figure 1. Structural characterization of glucogalactan polysaccharide (GA) extract by FT-IR.

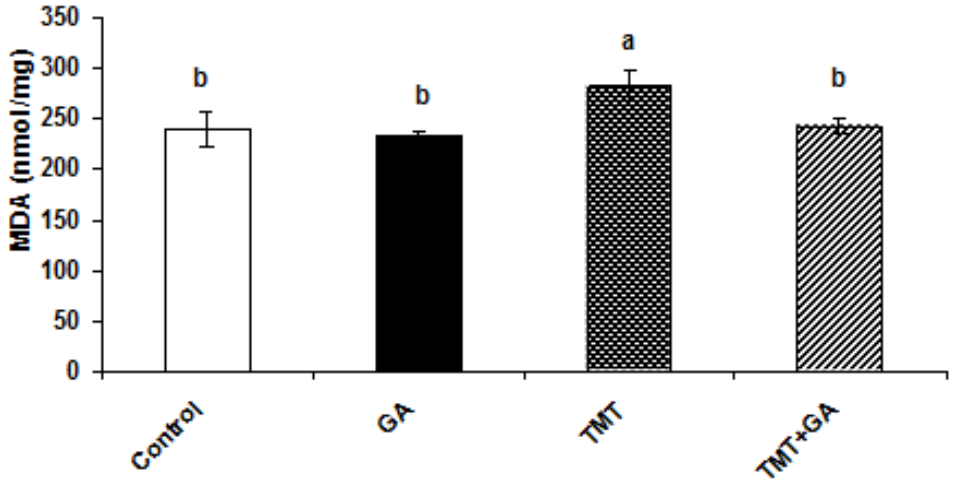

Figure 2. Impact of Trimethyltin (TMT) or glucogalactan polysaccharide (GA) on malondialdehyde (MDA): **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 3.347, P<0.05.

increase (p< 0.05) of MDA contents in brain tissues of group of rats administrated with TMT compared to control group (282.69±15.6 and 239.32±17.42, respectively) (Figure 2). The elevation of MDA was significantly (p< 0.05) decreased in group of rats administrated with TMT and treated with GA (243.15±9.50). On the other hand, there was significant (p< 0.05) decrease in the total antioxidants in brain tissues of group of rats administrated with TMT compared to control group (22.75±4.70 and 38.25±2.46, respectively); and this led to close to normal in the treated group (39.15±3.53) (Figure 3).

Effect of GA on NO and AchE in control and experimental groups

The results revealed that TMT reduced NO and AchE concentrations in brain tissues compared to the control group. In contrast, the results indicate significant (p<

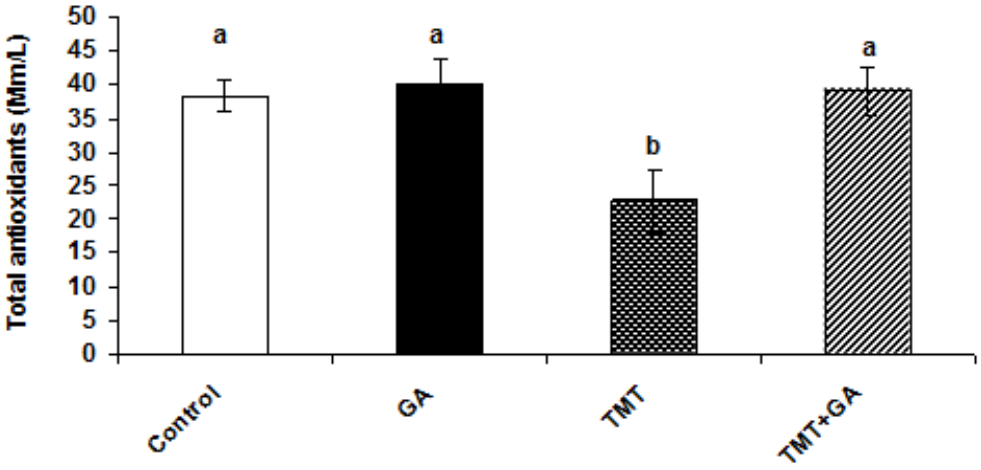

Figure 3. Effect of treatments on total antioxidants in brain of rats: **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 4.46*, P<0.05.

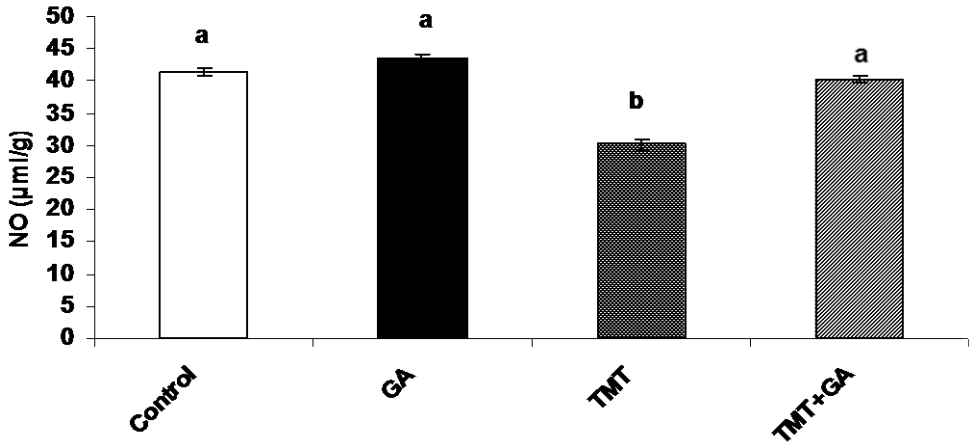

Figure 4. Effect of treatments on NO in brain of rats:**a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 3.347, P<0.05.

0.05)increase of NO and AchE concentrations in brain tissues in group of rats administrated with GA after 48 h of TMT; and this concentration increased to close to normal group (Figures 4 and 5).

Effect of GA on neurotransmitters in control and experimental groups

The NA, DA and 5-HT concentrations in brain tissues were investigated. The results reveal that the concentrations of these neurotransmitters decreased significantly (p< 0.05) in group of rats administrated with TMT as compared to control group. These concentrations increase significantly (p< 0.05) in group of rats treated with GA for 28 days to close to normal as compared with group of rats administrated with TMT only (Figure 6a, b and c, respectively).

Effect of GA on tissues homocysteine and HSP70 in control and experimental groups

Homocysteine and HSP70 concentrations in brain tissues of the present study showed significant increase (p< 0.05) in group of rats administrated with TMT compared to control group, and this increase normalized significantly (p< 0.05) in group of rats administrated with TMT and treated with GA compared to group of rats administrated with TMT only (Figures 7 and 8, respectively).

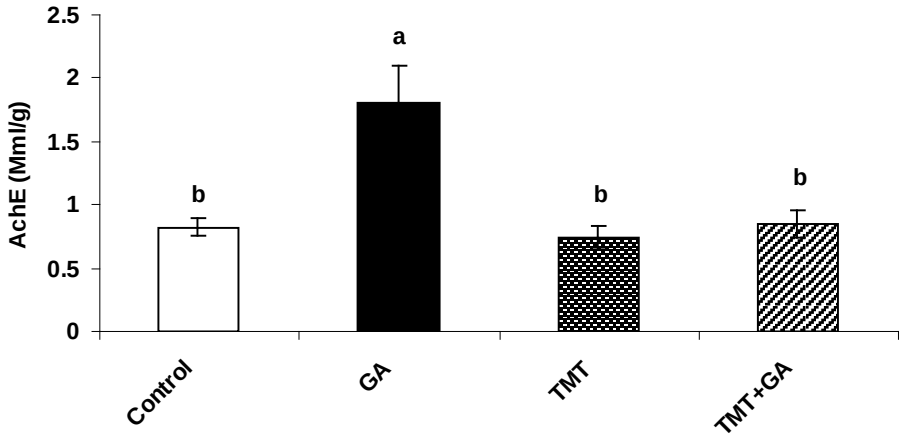

Figure 5. Effect of treatments on AchE in brain of rats: **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 9.638, P<0.05.

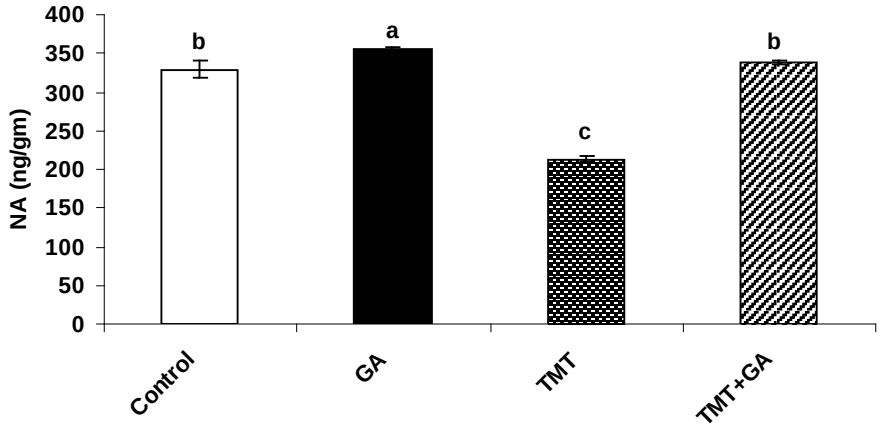

Figure 6a. Impact of treatments on NA in brain tissues of rats: **a, b and c:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 28.09, P<0.05.

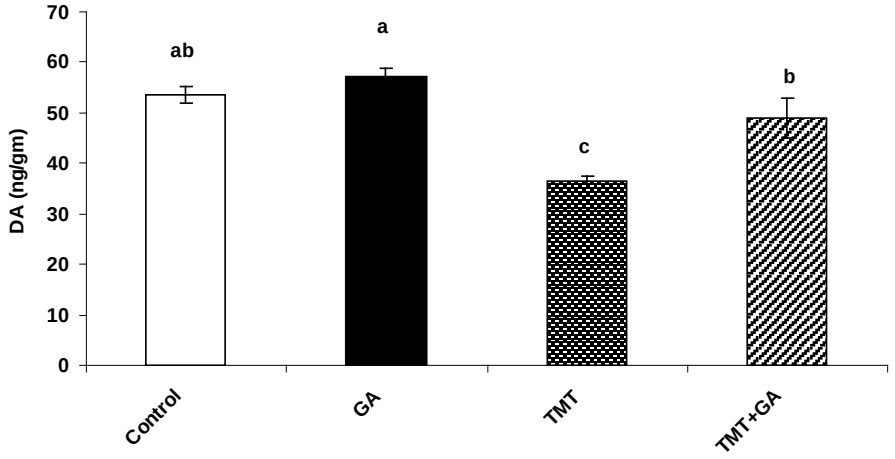

Figure 6b. Impact of treatments on DA in brain tissues of rats: **a, b and c:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 16.70, P<0.05.

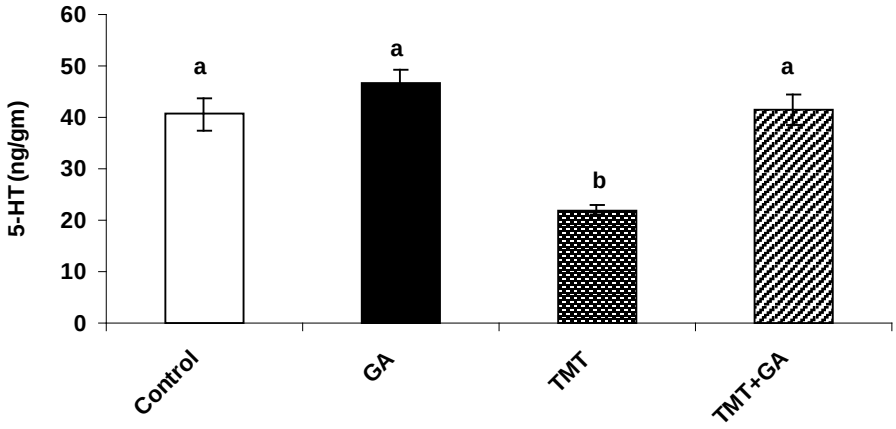

Figure 6c. Impact of treatments on 5-HT in brain tissues of rats: **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 18.52, P<0.05.

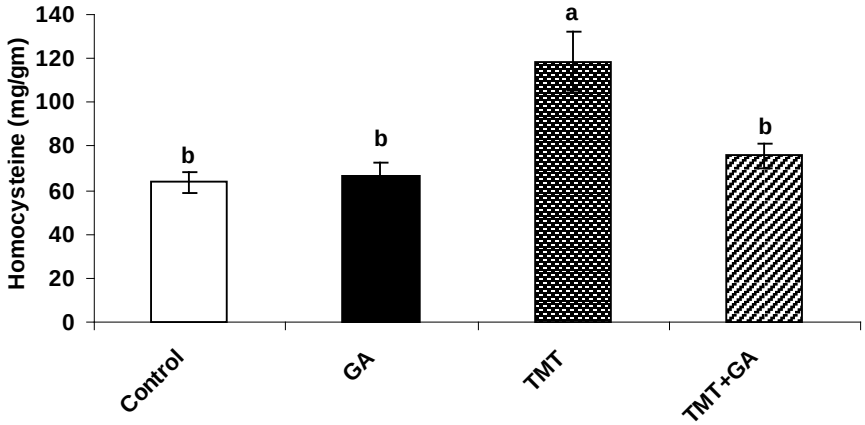

Figure 7. Impact of treatments on homocystein in brain tissues of rats: **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 9.64, P<0.05.

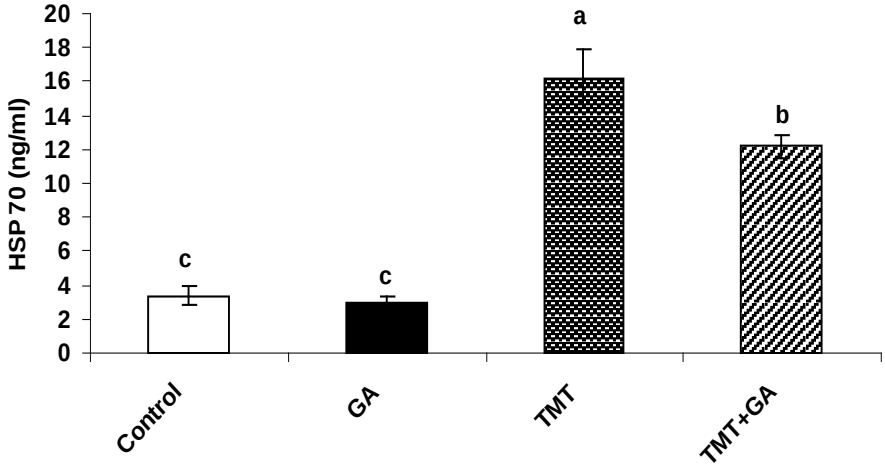

Figure 8. Impact of treatments on HSP70 in brain tissues of rats: **a and b:** Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; F = 28.36, P<0.05.

Table 1. Effect of GA on genomic DNA in the control and experimental groups.

Groups	Control	GA	TMT	TMT+GA	F
Tail DNA (%)	1.79±0.11^{c}	1.95±0.18^{c}	5.90±0.03^{a}	3.37±0.72^{b}	27.38*
Tail length(µm)	1.76±0.07^{c}	1.96±0.01^{c}	5.96±0.72^{a}	3.05±0.6^{b}	30.63*
Tail moment(Units)	3.16±0.30^{c}	3.82±0.25^{c}	35.80±3.24^{a}	8.06±1.42^{b}	77.41*
Untailed DNA (%)	95.67±1.53^{a}	96.33±0.58^{a}	77.33±3.06^{c}	90.33±3.51^{b}	38.26*

Data expressed as mean ± SE. a, b and c: Statistically significant from control or experimental groups at P<0.05 using one-way ANOVA followed by Duncan as a post-hoc test; * F (P<0.05).

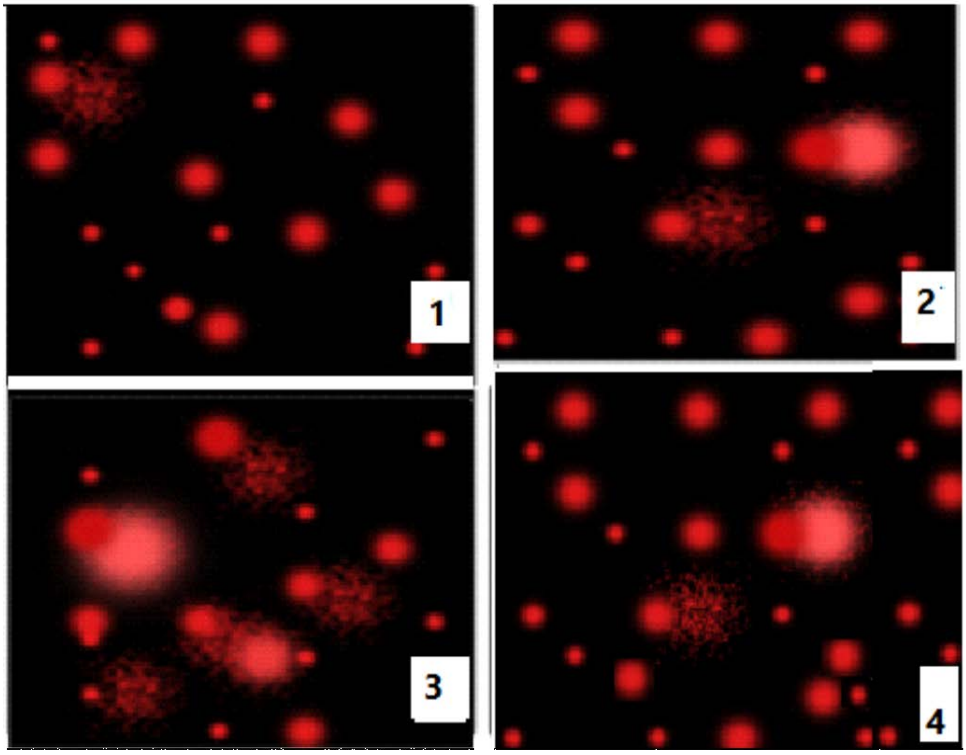

Figure 9. Photographs showing the effect of GA treatment against TMT-induced DNA damage in the brain of rat as measured by the comet assay represented by Tail length (µm) and % of DNA damage in the brain cells from control **(1),** GA only treated rats **(2),** TMT treated rats **(3)** and TMT + GA treated rats **(4)**.

DNA damage detected by the comet assay

Concerning the brain genotoxic potential of TMT using the comet essay, there was a significant increase in the tail length of DNA, tail intensity (DNA %) and tail moment in the TMT treated rats compared to the control (Table 1 and Figure 9). On the other hand, the treatment with GA significantly decreased DNA tail length, intensity and moment as compared to the TMT treated rats (Figure 9).

Histopathological findings

The present study revealed that there was no hisopathological alteration, and the normal histological structures of the meninges, cerebral cortex, cerebral striatum and hippocampus were recorded in the control group administrated with GA (Figures 10a, b and c, respectively). In group of rats administrated TMT, the cerebral striatum showed desquamation in the lining endothelium of the congested blood vessels with diffuse gliosis (Figure 10d). Diffuse gliosis was noticed also in between the neuronal cells of the hippocampus (Figure 10e). There was focal area of neuronal degeneration with gliosis in the cerebral striatum (Figure 10f). Encephalomalacia with vacuolization was detected in the cerebellum (Figure 10g); while group of rats administrated TMT and treated with GA showed significant improvement

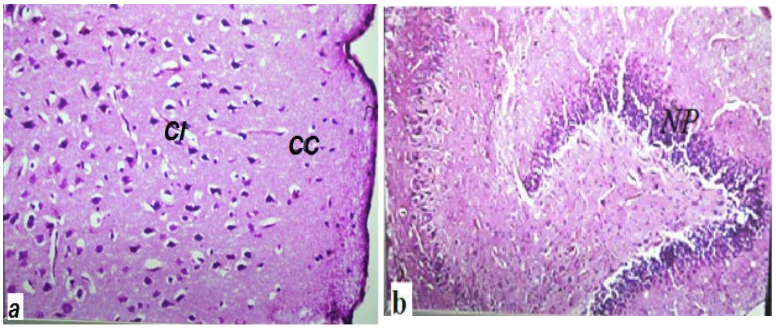

Figure 10a, b: Photomicrograph of a section in brain of control rat, there was no hisopathological alteration and the normal histological structure of the meninges, cerebral cortex, cerebral striatum and hippocampus.

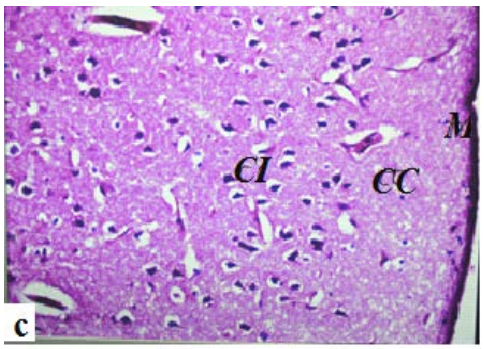

Figure 10c: Rats treated with GA only there was no histopathological alteration.

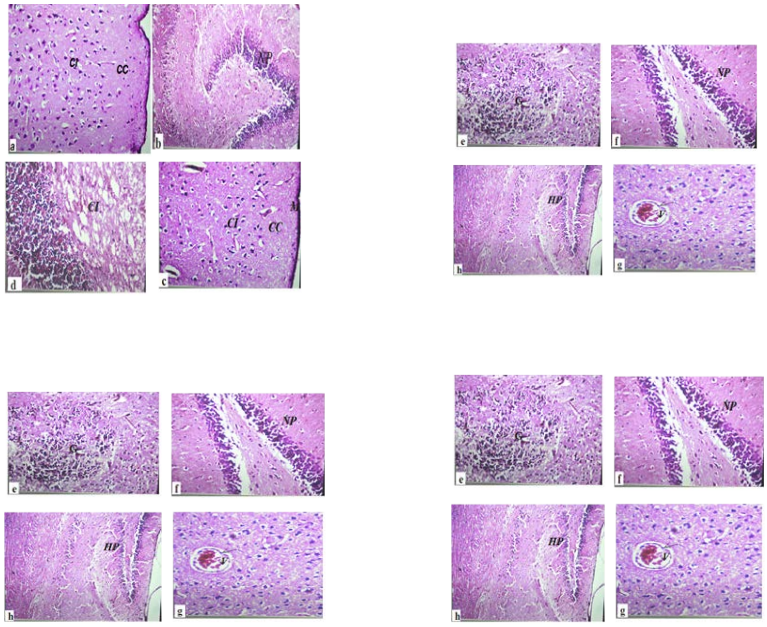

Figure 10d, e, f and g. Histopathology of brain TMT only treated rats. **(d).** Photomicrographs showing the cerebral striatum showed desquamation in the lining endothelium of the congested blood vessels with diffuse gliosis. **(e).** Diffuse gliosis was noticed also in between the neuronal cells of the hippocampus.**(f).** There was focal area of neuronal degeneration with gliosis in the cerebral striatum. **(g).** Encephalomalacia with vacuolization were detected I the cerebellum.

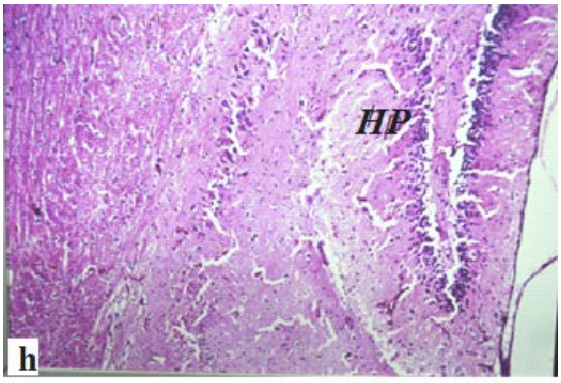

Figure 10h. Treated group with TMT+ GA with significant improvement in the brain tissues where the cerebral striatum showed slight congestion in the blood vessels.

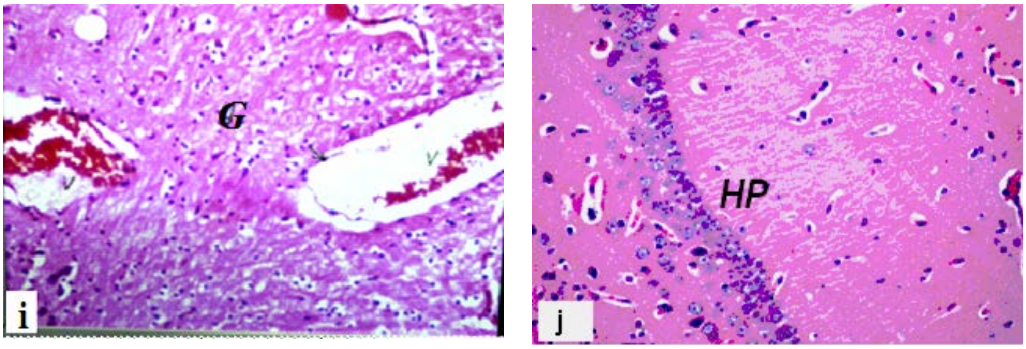

Figure 10i and j. Treated group with TMT+ GA. **(i).** The cerebral striatum showed congestion in the blood vessels; while the hippocampus was histological normal. **(j).** Brain of rat in group four (TMT +GA) showing normal histological structure of the hippocampus (HP).

in the brain tissues where the cerebral striatum showed slight congestion in the blood vessels (Figure 10h), and the hippocampus was histologically normal (Figure 10i and 10j).

DISCUSSION

Trimethyltin chloride (TMT) is a potent neurotoxicant that causes selective neuronal death specifically localized in the limbic system and particularly in the hippocampus of the mammalian brain (Chang, 1990). The molecular mechanisms by which TMT induces selective neuronal death are still not conclusively clarified; different pathogenetic pathways, probably acting differently *in vivo* and *in vitro* models, seem to be involved, including neuroinflammation, intracellular calcium overload, and oxidative stress (Geloso et al., 2011). In the present study, TMT acute exposure induced brain cell apoptosis and oxidative damage to rat brain, as evidenced by significant rise in brain MDA (an end product of lipid peroxidation) levels; it also induced significant reduction in total antioxidant activities. The increase in tissue MDA levels in the present study was observed in the group that was administered TMT alone. This demonstrates that lipid peroxidation has developed. The occurrence of a significant increase in brain MDA levels compared to the control group is also indicative of damage to have been caused in the brain tissues examined, as a result of free radicals generated by TMT. The consequence of oxidative stress to nervous tissue is many, as brain is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption. Oxidative stress induces many damaging processes in stress disorders such as mitochondrial dysfunction, dysregulation of calcium homeostasis, disruption of energy pathway (Amoroso et al., 2000), damage to neuronal precursors, impairment of neurogenesis (Papadopolos et al., 1997). After TMT administration, rats develop extensive lesions in the CA, typically localized in the CA3/Hilus and also involving CA1,

while granular neurons in the DG are generally spared (Moradali et al., 2007). Neuronal death shows a delayed onset (two days after treatment) and progressively worsens: it develops over three weeks (Geloso et al., 2011), probably on account of the high affinity of rat hemoglobin for TMT, involving CA3 earlier and more severely than CA1(Corvino et al., 2012) . TMT activates different pathogenic mechanisms leading to cell death; the approach based on gene profiling examination appears to be promising, since it provides a comprehensive snapshot of the molecular scenario. Several studies have demonstrated behavioral abnormalities such as increased seizure susceptibility, aggression, and learning impairment after TMT-induced neurodegeneration, which is consistent with results of this study (Bernaś et al., 2006). GA was found to be a highly potent antioxidant that could inhibit free radical generation in the brain. It has been shown that it inhibits the lipid peroxidation and prevents cell death induced by oxidative stress. Recent research has shown that some polysaccharides rich in mannose, glucose or galactose play important roles as free radical scavengers, ferrous metal ion chelators and reducers for the prevention of oxidative damage in living organisms (Tian et al., 2011).

The glucogalactan chains were composed of (1→6) (1→5)- and (1→5,6); the biologically active polysaccharides mainly in the form of β -D-glucans are extracted from these organisms, and also galactomannans, heteroglycans, and fucogalactans (Bi et al., 2013). Mushrooms included in the same genera show more similarities in their composition, including the structure of carbohydrates (Carbonero et al., 2008). In the present study, an increase in the levels of lipid peroxidation was found in TMT group and this was significantly reduced after treatment with GA. Recently, it has been found that polysaccharides have many potent biological and pharmacological activities, including immunostimulation as well as anti-tumor, anti-virus, anti-inflammatory and hypoglycemic activities (Wang et al., 2010). Mushroom polysaccharides have recently become attractive as food and as sources for the development of drugs. Hence, antioxidant status has been suggested as a useful tool in estimating risk of oxidative damage induced neurodegeneration. The present study shows that the treatment with TMT decreased total antioxidant activities. This observation is in agreement with Jung et al. (2013). While, the administration of GA to TMT – treated rats showed restoration of total antioxidant capacity towards normal. Several polysaccharides from fungi have exhibited strong antioxidant effects that are relevant to their health-protecting functions (Tseng, et al., 2008). The free radical of DPPH is stable and widely used to evaluate the free radical scavenging ability of natural compounds. The DPPH radical-scavenging activity is conceivably due to a hydrogen-donating ability.

In the present study, the biochemical analysis of brain tissue revealed that administration of TMT induced significant increase in homocysteine and AchE levels and significant decrease in NO level. These results agree with previous findings that chronic experimental hyperhomocysteinemia lead to oxidative stress (Chao and Lee, 2000), decrease in NO bioavailability ((Volman et al., 2010) and increase in brain AchE (Monteiro et al., 2005). The oxidative stress results from auto-oxidation of homocysteine and generation of free oxygen radicals (Hartl et al., 2011). Improvement in these parameters was observed in the group which was administered with GA treatment.

In this study, we evaluated the capacity of the mushroom extracts to stimulate the production of the neurotransmitters (NA, DA and 5 HT) in brain tissues. Some polysaccharides or polysaccharide–protein complexes from mushrooms are able to stimulate the non-specific immune system and to exert antitumor activity through the stimulation of the host's defense mechanism (Reshetnikov et al., 2001). It is now well established that NO is a physiological mediator of the central nervous system. The role of NO in developing brain remains poorly understood, but it seems to be involved in the regulation of cerebral blood flow, and in memory acquisition. In fact, NO appears to be a double-edged sword, simultaneously neurotoxic and neuroprotective. Numerous experimental studies demonstrated the deleterious effects of nitrogen reactive species accumulation in ischemic-reperfusion cerebral injury through depletion of energy, lipid peroxidation, protein nitrosylation, DNA alterations and increased permeability of the blood brain-barrier (Arul and Konduri, 2009). Hypoxia-ischemia results in inflammation, especially in the developing white matter. High concentrations of NO and peroxynitrite produced locally by activated microglia may become toxic to neurons and immature oligodendrocytes *in vitro* (Li et al., 2005). It is also well known that hypoxia-ischemia results in the accumulation of extracellular glutamate, inducing the excitotoxicity cascade that causes neuronal death. More or less effective neuroprotection can be achieved by using NOS inhibitors that inhibit nNOS at the early phase and iNOS during the reperfusion of hypoxic insult (Margaill et al., 1994). An increase in brain infarct volume has been reported in sheep and rat when NO production is decreased by NOS inhibitors (Rosenberg et al., 1999). NO seems to be beneficial to the brain mostly through its vasodilator effects, and has proangiogenic effects potential.

In the current study, TMT induced significant increase in HSP70. This result agrees with Anderson et al. (1997). On the other hand, the administration of GA showed significant decrease in HSP70, but still greater than normal. Hsp70 proteins can act to protect cells from thermal or oxidative stress. These stresses normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, Hsp70 prevents these partially denatured proteins from aggregating, and

allows them to refold. Our data shows that TMT induced statistically significant increase in DNA damage which represented by tail moment in brain tissues. The DNA breaks that are detected in the comet assay and cytogenetic analysis may be resulted from the oxidative stress and enhancement of the intracellular generation of reactive oxygen species (ROS) formed by TMT (Sergent et al., 1999). These ROS can damage DNA and division of cells with unrepaired or misrepaired damage leading to mutations. This DNA damage could also originate from apoptotic cells. Several laboratories have reported that the onset of apoptosis can give comet images whose cell aspect and tail parameter values are the same with cells of moderate DNA damages (Choucroun et al., 2001).

Conclusion

Mushrooms can be viewed as an important source of bioactive polysaccharides. The most common pattern consists of a β-linked glucose backbone displaying branches at certain sugar residues; nevertheless, other polysaccharides, such as galactans. These carbohydrates have been considered as biological response modifiers due to their ability to ameliorate the neurodegenerative induced by TMT through the improvement of some biochemical parameters in brain tissues and serum such as MDA, neurotransmitters, homocysteine and % tail DNA additionally, total antioxidant capacity and NO. Changes determined in the parameters examined may be reliable indicators of neurodegenerative impairment. Further studies are needed in order to proof its efficacy in the treatment of Alzheimer as a result of neurodegenerative *in vivo*.

Conflict of interests

The authors did not declare any conflict of interest.

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