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African Journal of Biotechnology

Full Length Research Paper

Responses of soybeans (*Glycine max* (L.) Merrill) associated with variable plant density stress applied at different phenological stages: Plasticity or elasticity?

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The study evaluated developmental responses associated with plant population density stress applied at different phenological phases and effects on grain yield in determinate and indeterminate soybean (*Glycine max* L.). A split-split plot design with four replications, with variety (main plot), plant density (sub plot) and thinning time (sub-subplot) was adopted. Two determinate genotypes (Lukanga and SC Semeki) and an indeterminate type (Mwembeshi) were used. Plant density stress was imposed by planting at supra optimal densities (700, 600 and 500 K plants ha^{-1}) (K representing 1000) and stress was removed by thinning to the recommended density (400 K plants ha^{-1}) at different phenological stages. Plant density had little effect on grain yield. Thinning time influenced root to shoot ratio, number of grains per pod, yield and harvest Index (HI). Lukanga had the highest grain yield (2.43 tons ha^{-1}), followed by Mwembeshi (1.95 tons ha^{-1}) and lastly SC Semeki (1.17 tons ha^{-1}). Lukanga exhibited reproductive plasticity, while SC Semeki showed vegetative plasticity. Mwembeshi an indeterminate type suggested non-plastic or 'elastic' response. The lack of effect on planting density exemplified by constant yield at different plant densities suggests that maintaining low seed rates is more economical given the high cost of seed.

Key words: Partitioning, determinate, indeterminate, reproductive plasticity, vegetative plasticity.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill)**,** is an oilseed crop that is produced worldwide in varying environments (FAO, 2016). It is one of the field row crops that manifest general growth characteristic of having phenotypic plasticity, a concept which enables plants to counter environmental stresses (Mataa and Sichilima, 2019). Resource competition imposed by high plant population density results in a negative growth relationship per unit plant as recorded by Pacala and Tilmant (1994). This notion was confirmed by Ibrahim (2012) and Li et al. (2019) who observed that as competition for environmental resources (e.g. light, nutrients, moisture

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among others) increased, through increased plant population density, there were decreases in individual plant biomass accumulation and this phenomenon was associated with assimilate allocation plasticity (Rondanini et al., 2017; Ibrahim, 2012).

In row crops, where intraspecific competition is at play, plants tend to utilize their biomass allocation plasticity capacities in order to 'win' (Yang et al., 2019). Yang et al. (2019) suggested that in severe asymmetric competition, plants allocated more of their biomass to plant parts that gave them a competitive edge in accessing resources in limitation (such as moisture and light). Hence plants may partition more assimilates to the below- ground tissues (like roots) and to support structures (e.g. stems and petioles). When competition is asymmetric, it results in weaker plants being out competed and the vigorous plants induced into positive morpho-physiological responses (Mellendorf, 2011; Rondanini et al., 2017). Park et al. (2003) described that asymmetric competition results when smaller plants get a disproportionately lower share of the available resources from the environment to their detriment because larger plants have had excessively higher share of the resource in question to the point of limiting growth and productivity of the surrounding plants (Yang et al., 2019).

As suggested by Bradshaw (1965), phenotypic plasticity induces a positive morphophysiological response that raises the plants' resource acquisition competitiveness per unit photoassimilate produced, thereby enhancing the plants' development capacity despite the resource limitation (Craine and Dybzinski, 2013). This concept shows that the plasticity is functional at some critical phenological stage of a plant (Mataa and Sichilima, 2019). Thus, early relief (in the developmental cycle) of the induced stress due to high plant population density (by thinning of weaker plants), theoretically builds the plants yield capacity (Mellendorf, 2011; Rondanini et al., 2017). Plasticity responses to adverse environmental cues that are restricted to vegetative phenotypic traits are referred to as vegetative plasticity (Rondanini et al., 2017). In contrast, reproductive plasticity was described by Sultan (2003) as adjustments to phenotypic traits that directly affect the reproductive success of a plant in response to various environmental stress signals.

During the process of plant development, many physiological processes that occur are controlled by plant hormones available only at specific growth stages (Maggio et al., 2018). Accurate determination of the different growth stages is critical for growth environment manipulation to influence plant development and determine potential yield loss from environmental stresses (Wright and Lenssen, 2013; Agalave, 2017). In soybeans however, the phenological stage at which plants can exhibit plasticity responses after thinning when exposed to high plant population density is not well known.

According to classical phenotype plasticity (Bradshaw, 1965; Sultan, 2003), two forms of plasticity are recognized -morphological and physiological - each with different mechanisms, resource costs and ecological implications (Bradshaw, 1965; Murren et al., 2015). The first form is meristematic in character and involves the replacement of existing tissues by new plant parts with different phenotypic characteristics: it appears to represent a high-cost solution to a change in environment (Grime and Mackey, 2002). The second- physiological plasticity- occurs in differentiated tissues and is associated with visually imperceptible changes in properties brought about by reversible sub-cellular rearrangements: here the costs are lower and the response can be much more rapidly achieved than in morphological plasticity (Mooney and Gulman, 1979; Grime and Mackey, 2002).

In an earlier study (Mataa and Sichilima, 2019), it was observed that soybean responses to stress were plastic with a range of alternative pathways or phenotypes. Additionally, stress caused plants to adopt certain phenotypes and generally no recovery or change was possible thereafter. Furthermore, these responses appeared to be dependent on the genotype and phenological stage at which stress was applied.

In this paper, we examined whether other responses were possible and/or whether plants can exhibit non-plastic or 'elastic' responses whereby previously stressed plants were able to recover and achieve normal yield. The main objective of the study was to determine the extent to which plant density and thinning time influence crop development and yield in soybeans, thus the study evaluated more detailed environmental responses in soybeans. Determinate and indeterminate soybean varieties were subjected to a wider range of supra-optimal planting density stress which was removed at different phenological stages and the recovery or elasticity was monitored.

MATERIALS AND METHODS

Location

The experiment was conducted in the 2016 planting season at Mansa Research Station, Zambia (latitude 11° 14' 27.0" S and longitude 28° 57' 23.2" E) which is located in Agro-ecological Region III that is characterized by high rainfall of about 1200 mm (Chileshe and Chirwa, 1990). The site which had been fallow for two years have an elevation of 1231 m above sea level. The soils were classified as Acrisols according to the Food and Agriculture Organization (FAO, 2001) classification system. The soils were acidic with a pH of 4.4.

Plant materials

The following soybean varieties were used: Mwembeshi, an indeterminate type, self nodulating and an early maturing variety

developed by IITA; Lukanga a medium maturing, determinate and non-self nodulating variety from ZamSeed Company; SC Semeki, a determinate type from SeedCo and non-self nodulating variety with a medium maturity (SeedCo, 2015; Chigeza et al., 2019).

Experimental design and treatments

A split-split plot design with variety, planting density and thinning time assigned to the main plot, subplot and sub-subplot respectively was used in the study. The smallest unit plot measured 1.5 m by 1.5 m. The recommended plant density for Zambia of 400,000 plants ha^{-1} (or 400 K plants ha^{-1}, where K denote 1000) was used as a control (Mitti, 1995). Three supra normal plant density levels (500 K; 600 K and 700 K plants ha^{-1}) were adopted at planting and thinned down progressively to the 400 K control density level at V_0, V_4, R_1 and R_8 phenological stages (Wright and Lenssen, 2013). Hence the experimental variants were: V_0 was at planting before emergence; V4 (five nodes with 4 unfolded trifoliate), R_1 was the onset of the reproductive growth phase, where the plant has developed at least one open flower at any node and R_8 was when the plant reached full maturity and at least 95% of the pods had attained their full maturity color (Fehr et al., 1971; Wright and Lenssen, 2013). In practice, V_0 was maintained with no thinning.

Cultural practices

Standard soybean production guidelines were followed (Mitti, 1995). Briefly, prior to seeding, the land was ploughed and subjected to harrowing. Each plot was applied with a stimulative dose (33 kg ha^{-1}) of granular D-compound (NPK 10:20:10+6S) fertilizer as recommended. In the early stages of crop development, the nitrogen-fixing systems is inoperative, hence the need to apply an external nitrogen (N) to meet the plants' demand (Oyatokun and Oluwasemire, 2014). Planting was done on 9th January, 2016. Seeds were drilled by hand and thus the spacing ranged from 2.5 to 7 cm within rows to achieve the different planting densities. In all treatments, a 30 cm distance between rows was maintained. The seeds were inoculated with rhizobia (*Rhizobium leguminosarum)* at the time of seeding; hand weeding, pests and disease control were done as the need arose; supplemental irrigation was done only when the rains were inadequate and soil moisture was deemed to be below field capacity. Further, harvesting was done by hand approximately 120 days after sowing (DAS) when the crop had reached physiological maturity (8th to 10th May 2016).

Data collection

The following vegetative parameters were monitored.

Plant height: Using a hand rule, five randomly selected plants were placed in a sub-subplot; thereafter, plant height were measured from the base to the uppermost shoot tip and expressed as a mean of the five individual plants at R1 and R8 growth stages. Using destructive sampling, five plants were harvested carefully from the border roles, fresh weights were taken and thereafter the samples were air-dried under shade (for about 2 weeks) to constant mass and then reweighed to determine total dry biomass weight (at V4 and R8).

Root-shoot weight ratio: The fresh and dry mass of the below ground and above ground dry biomass from five randomly selected plants were taken at V4 phenological stage. Means of the five plants were used to compute the root and shoot ratios expressed as percentages (at V4).

The reproductive parameters that were monitored include

Number of pods per plant: This was determined by taking counts of number of pods at maturity (R8) of the five randomly selected plants.

Grains per pod: This was determined at harvest by counting the number of grains from five randomly selected plants and expressed as a mean value.

100-grain weight: This was measured by taking a triplicate sample of 100 grains per subplot of soybeans at harvest to come up with a representative mean weight.

Grain yield: This was determined from each plot by weighing the total grains harvested in the net plot (excluding guard rows) and expressed in tons per hectare.

Harvest index (HI): This was computed at harvest by dividing grain yield with the total dry biomass yield.

Statistical analysis

Data was analyzed using GenSTAT (version 18) (VSN, 2015) and graphical illustrations were generated using Microsoft Excel. Data were subjected to analysis of variance, correlation and multiple regression. Where differences were considered significant, mean separation was done using the least significant differences method.

RESULTS

Yield was highly influenced by variety ($p \leq 0.001$). Biomass at V4 was very significantly influenced by variety (G) ($p \leq 0.01$) (Table 1). Plant heights, biomass at R8, grains per pod, number of pods per plant and harvest index were significantly influenced by variety ($p \leq 0.05$). Plant density (D) had no significant effect on most parameters ($p > 0.05$) except for biomass at R8, 100-grain weight and harvest index ($p \leq 0.05$). Thinning (T) influenced significantly root to shoot weight, grains per pod and harvest index ($p \leq 0.05$). Thinning stage affected yield very significantly ($p \leq 0.01$) while root to shoot ratio, grains per pod and HI were significantly affected ($p \leq 0.05$). There were significant interactions between treatment factors (Table 1).

Vegetative parameters

Plant height

The main factor showed that Lukanga had significantly taller plants followed by Mwembeshi and SC Semeki was the shortest (Table 2). Density and thinning did not show single factor effects. The interactive effects of variety with density and thinning were significant ($p < 0.05$), particularly on Lukanga at all levels of density and for thinning at V0, V2 and R1 phenological stages (Table 3)

Table 1. Summary ANOVA table showing significance of different sources of variation.

Source of variation	Plant height (R1) [y]	Plant height (R8) [x]	Biomass (V4) [z]	Biomass (R8) [x]	[w] Root: shoot	Grains per pod	Pods per plant	100- Grain weight	Grain Yield	Harvest index
Variety (G)	*	*	**	*	ns	*	*	ns	***	*
Density (D)	ns	ns	ns	ns	ns	ns	ns	*	ns	*
Thinning time (T)	ns	ns	ns	ns	*	*	ns	ns	**	*
GxD	*	*	*	*	*	*	*	ns	*	**
GxT	*	*	**	*	*	*	*	ns	***	*
DxT	ns	*	***	*	*	*	*	**	***	*
GxDxT	**	ns	***	*	*	*	*	*	***	*

Factor significance; *** highly significant ($p \leq 0.001$); **very significant ($p \leq 0.01$); * significant ($p \leq 0.05$) and ns- non-significant. [z] Vegetative stage V4, [y] Reproductive stage R1, [x] Reproductive stage R8, [w] Root: shoot ratio- determined at vegetative stage V4.

Table 2. Treatment effects on selected parameters during the soybean vegetative and reproductive growth phases.

Factor		Vegetative parameter					Reproductive parameter				
		Plant height (cm)		Biomass (ton. ha^{-1})		[w] Root: Shoot	Grains per pod	Pods per plant	100-Grains weight (g)	Yield (ton. ha^{-1})	HI (%)
		R1	R8	V4	R8						
Variety (G)											
Lukanga	(G_1)	29.30	34.32	1.99	5.81	10.87	2.41	12.24	13.27	2.43	37.99
Mwembeshi	(G_2)	24.63	33.14	1.72	5.44	9.00	2.32	16.35	13.56	1.95	35.24
SC Semeki	(G_3)	24.42	31.48	2.76	2.90	11.24	2.24	13.73	13.42	1.17	34.56
Lsd (G)		*4.13*	*2.12*	*0.44*	*1.85*	*2.71*	*0.10*	*3.87*	*0.46*	*0.24*	*3.28*
Planting density (D)[z]											
500	(D_1)	25.29	33.01	2.20	4.24	10.56	2.34	14.38	13.29	1.83	36.47
600	(D_2)	27.35	33.56	2.30	5.70	11.67	2.34	13.72	13.41	1.89	36.13
700	(D_3)	25.72	32.37	1.97	4.21	8.88	2.30	14.21	13.55	1.83	35.20
Lsd (D)		*2.98*	*2.35*	*0.40*	*1.42*	*3.71*	*0.07*	*2.19*	*0.26*	*0.21*	*1.07*
Thinning stage (T)											
V0[v]	(T_1)	25.38	32.74	2.08	5.25	9.50	2.34	14.45	13.27	2.02	36.95
V4[z]	(T_2)	27.02	33.08	2.18	4.73	9.25	2.31	13.64	13.44	1.86	34.53
R1[y]	(T_3)	25.81	32.96	2.33	4.43	9.78	2.38	14.81	13.38	1.67	36.80
R8[x]	(T_4)	26.26	33.14	2.03	4.45	12.95	2.26	13.53	13.57	1.85	35.45
Lsd (T)		*2.04*	*1.71*	*0.51*	*1.06*	*3.15*	*0.11*	*2.42*	*0.37*	*0.19*	*1.93*

[v] Thinning at planting [z] Vegetative stage V4, [y] Reproductive stage R1, [x] Reproductive stage R8, [w] Root: shoot ratio- determined at vegetative stage V4.

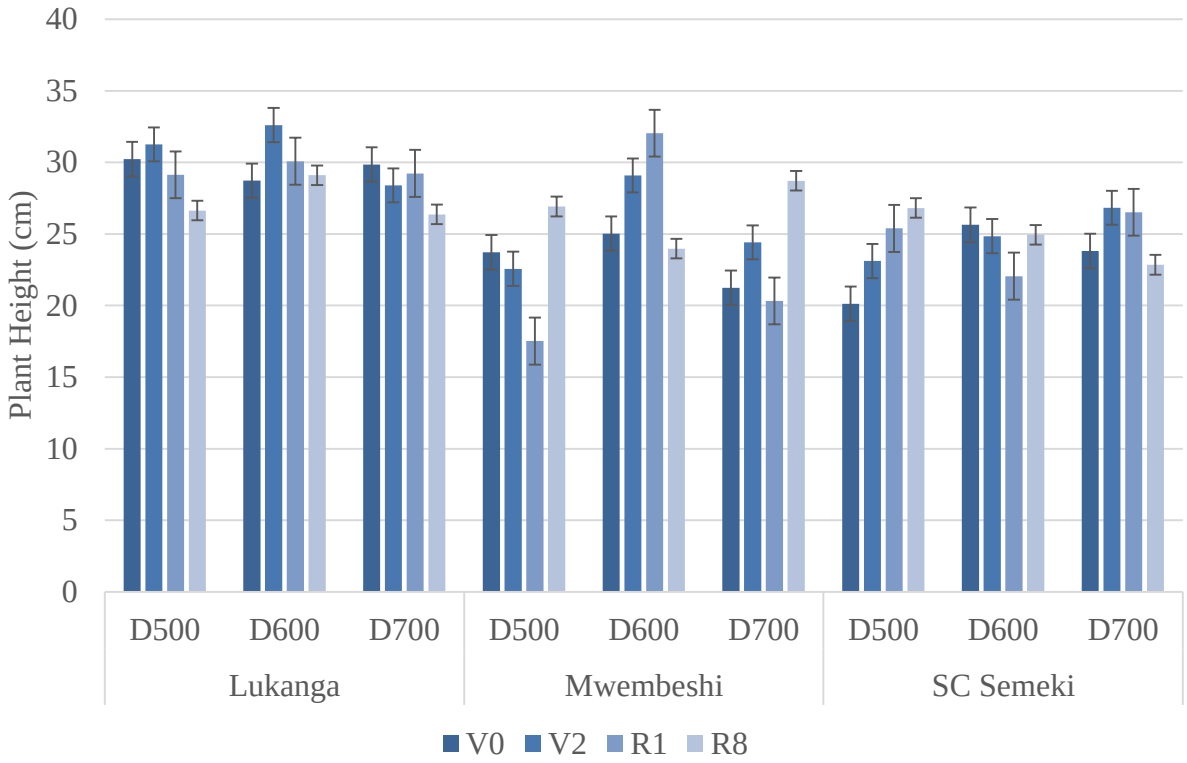

Figure 1. Plant height of different soybean cultivars planted at different plant densities and thinned at different phenological stages (that is, V0, V2, R1 and R8) as measured at R1. Vertical bars are standard errors.

when measurements were taken at the end of the vegetative growth phase (R1). The interaction of Lukanga with all levels of density and thinning at the maturity stage (R8) of the plants was significant. The interaction of Variety x Density x Thinning time at the R1 growth phase was very significant, specifically for Lukanga (32.61 cm) and Mwembeshi (32.04 cm) at density 600 K plants ha^{-1} which were thinned at V2 and R1 respectively (Figure 1).

Biomass

Early in the growth cycle at V4, SC Semeki had significantly higher biomass (2.76 tons ha^{-1}) than the other two varieties (1.8 tons ha^{-1}) (Table 2). But the opposite was true at R8, where Lukanga (5.81 tons ha^{-1}) and Mwembeshi (5.44 tons ha^{-1}) had significantly more biomass than SC Semeki (2.90 tons ha^{-1}). The main effects of density and thinning time were not significant on biomass. The results showed that all interactions were significant for biomass at both measurement stages (V4 and R8). An interesting contrast was that SC Semeki showed significant effects at all levels of density at the V4 stage, while Lukanga exhibited the same but at the R8 stage for all the density levels. Figure 2 shows the variety x density x thinning interaction where Lukanga, at 500 K plants ha^{-1}, had biomass that increased with delay in thinning. At 700 K plants ha^{-1}, the biomass reduced with delayed thinning. For Mwembeshi at 600 K and 700 K, biomass tended to decline with delayed thinning time. For SC Semeki at 500 K and 600 K plants ha^{-1}, biomass declined with a delay in thinning time. At 700 K plants ha^{-1}, it increased with the delay in the thinning time.

Root to shoot ratio

Only thinning time (T) showed a significant main effect on root: shoot ratio. Thinning time interacted significantly with variety and planting density (Table 3). The highest root to shoot ratio was seen in the combination of Lukanga with V2 thinning time (14.85%) followed by the density 500 K plants ha^{-1} with V2 thinning time (14.70%), and variety SC Semeki by density 600 K plants ha^{-1} (14.52%). In terms of variety x density x thinning time interactions (Figure 3), thinning for Lukanga at the V2 growth phase generally resulted in higher root to shoot weight ratio across planting densities. Density 600 K plants ha^{-1} resulted in the highest ratios for Lukanga and Mwembeshi when thinned at V2 and R1 respectively. SC Semeki demonstrated the propensity to reduce the root to shoot ratio for every successive thinning stage across

Table 3. Interactive effects of variety, planting density and phenological stage at thinning on vegetative parameters of soybeans.

Factor	Vegetative parameter					Reproductive parameter				
	Plant height (cm)		Biomass (ton. ha^{-1})		Root: Shoot ratio	Grains per pod	Pods per plant	100-Grains weight (g)	Yield (ton. ha^{-1})	HI (%)
	R1	R8	V4	R8						
G_1 x D_1	29.32	33.90	2.27	5.24	13.07	2.36	12.49	13.19	2.42	37.25
G_1 x D_2	30.13	34.62	2.25	6.49	10.15	2.49	12.41	13.11	2.30	39.51
G_1 x D_3	28.47	34.45	1.45	5.68	9.40	2.39	11.81	13.5	2.56	37.22
G_2 x D_1	22.68	32.23	1.72	3.87	7.90	2.38	15.55	13.5	1.83	36.27
G_2 x D_2	27.54	34.71	1.89	7.47	10.34	2.24	16.96	13.6	2.09	33.90
G_2 x D_3	23.67	32.47	1.53	4.98	8.74	2.35	16.55	13.57	1.93	35.57
G_3 x D_1	23.86	32.91	2.60	3.60	10.69	2.29	15.11	13.18	1.25	35.88
G_3 x D_2	24.38	31.33	2.76	3.13	14.52	2.29	11.80	13.51	1.27	34.98
G_3 x D_3	25.01	30.18	2.92	1.98	8.52	2.15	14.27	13.58	1.00	32.80
Lsd (G x D)	***5.46***	***3.73***	***0.68***	***2.53***	***5.65***	***0.14***	***4.57***	***0.55***	***0.36***	***3.40***
G_1 x T_1	29.61	34.13	1.67	5.70	8.88	2.38	12.62	13.10	2.68	39.62
G_1 x T_2	30.76	34.18	1.64	5.35	14.85	2.38	11.88	13.40	2.26	37.50
G_1 x T_3	29.48	34.76	2.46	6.19	9.02	2.48	13.43	13.12	2.32	38.05
G_1 x T_4	27.37	34.23	2.19	5.99	10.74	2.40	11.02	13.45	2.44	36.80
G_2 x T_1	23.33	32.84	1.73	4.52	8.46	2.42	17.23	13.46	2.14	35.07
G_2 x T_2	25.36	33.31	1.71	5.19	9.74	2.27	15.32	13.19	1.75	33.69
G_2 x T_3	23.30	33.02	1.10	6.74	9.44	2.35	17.05	13.83	1.94	36.91
G_2 x T_4	26.54	33.38	2.32	5.30	8.34	2.25	15.82	13.74	1.98	35.30
G_3 x T_1	23.20	31.90	3.58	3.07	12.00	2.23	13.50	13.24	1.25	36.17
G_3 x T_2	24.93	31.93	2.76	2.81	14.26	2.28	13.72	13.73	1.58	32.39
G_3 x T_3	24.66	30.44	2.67	2.83	10.04	2.32	13.93	13.19	0.74	35.43
G_3 x T_4	24.87	31.64	2.03	2.90	8.68	2.13	13.77	13.53	1.12	34.23
Lsd (G x T)	***4.78***	***3.12***	***0.84***	***2.27***	***5.22***	***0.18***	***4.94***	***0.68***	***0.35***	***4.07***
D_1 x T_1	24.69	33.49	2.03	4.04	10.95	2.33	14.43	13.36	1.90	38.33
D_1 x T_2	25.65	33.04	1.35	4.03	13.67	2.40	14.77	13.45	2.08	34.53
D_1 x T_3	24.02^{z}	31.20	2.58^{y}	4.03^{x}	8.58	2.40	13.05	12.99	1.90	36.29
D_1 x T_4	26.79	34.32	2.83	4.85	9.02	2.23	15.28	13.36	1.45	36.71
D_2 x T_1	26.47	32.92	2.66	5.03	9.58	2.37	13.95	13.42	2.51	35.90
D_2 x T_2	28.85	33.65	2.88	5.61	14.70	2.30	13.50	13.19	1.42	35.83
D_2 x T_3	28.06	35.84	2.03	7.56	13.35	2.40	16.85	13.83	1.67	37.03
D_2 x T_4	26.01	31.80	1.63	4.60	9.06	2.28	10.60	13.18	1.95	35.75
D_3 x T_1	24.97	32.45	2.29	4.23	8.81	2.33	14.97	13.03	1.66	36.63
D_3 x T_2	26.55	32.72	1.87	3.72	10.49	2.23	12.65	13.68	2.09	33.21

Table 3. Cont'd.

D_3 x T_3	25.36	31.17	1.62	4.16	6.57	2.35	14.52	13.32	1.42	37.08
D_3 x T_4	25.98	33.13	2.08	4.73	9.68	2.27	14.72	14.17	2.15	33.87
Lsd (D x T)	***4.18***	***3.40***	***0.85***	***2.09***	***5.88***	***0.17***	***4.17***	***0.61***	***0.35***	***3.06***

Reproductive stage R1, Vegetative stage V4 Reproductive stage R8. G, D and T denoting genotype, planting density and thinning stage respectively.

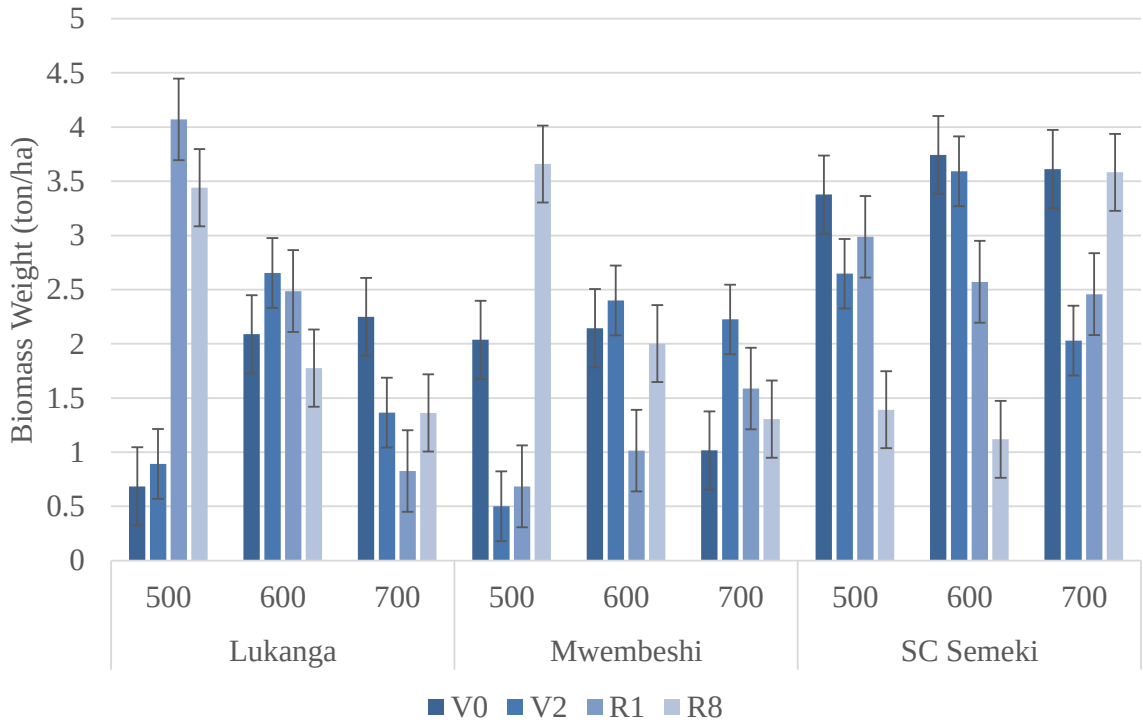

Figure 2. Biomass measured at V4 of different soybean cultivars planted at different plant densities and thinned at different phenological stages (that is, V0, V2, R1 and R8). Vertical bars are standard errors.

density levels. The highest ratio across all varieties and densities was observed in SC Semeki at density 600 K and thinning at V2 (21.68%) while the least was recorded in the same variety (G_3) but at the density of 700 K and thinning at R1 (5.42%).

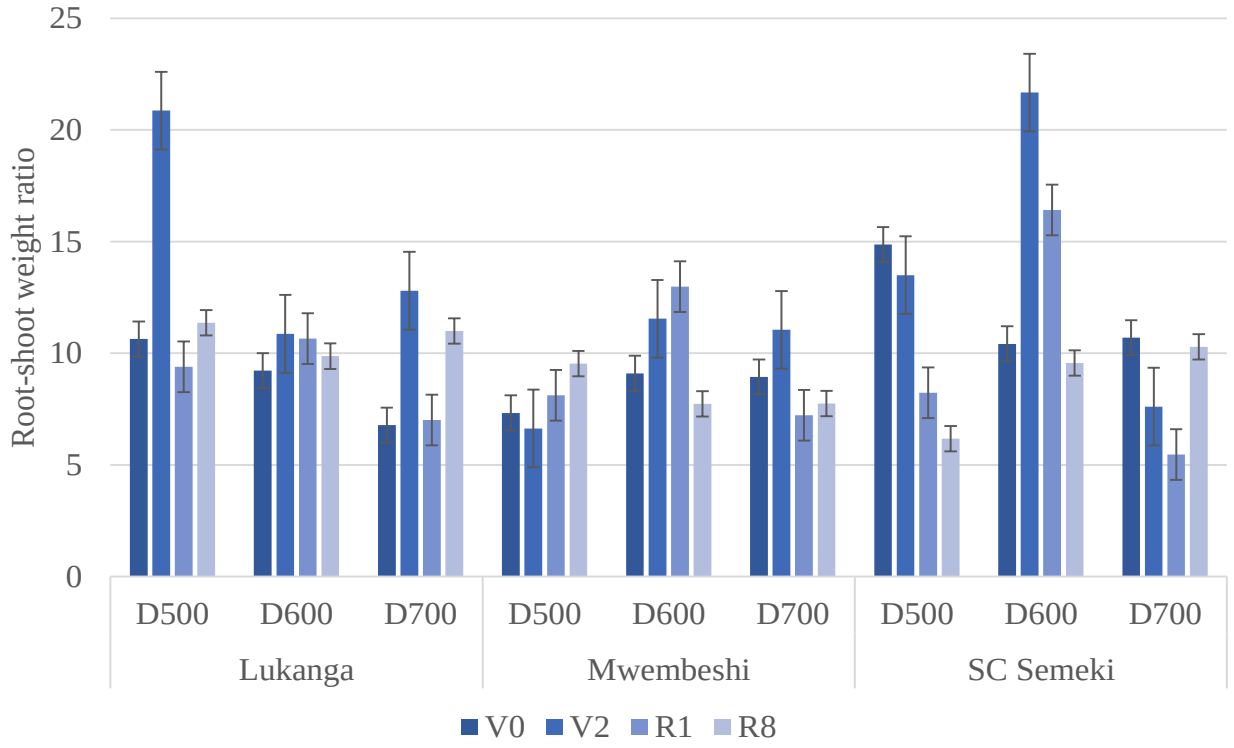

Figure 1. Root: Shoot weight ratio of different soybean cultivars planted at different plant densities and thinned at different phenological stages (that is, V0, V2, R1 and R8) as measured at R1. Vertical bars are standard errors.

Reproductive parameters

Number of grains per pod

As presented in Table 2, Lukanga had the highest number of grains per pod (2.41) in comparison to Mwembeshi (2.32) and SC Semeki (2.24). Plant density did not show significant main effect on number of grains, but thinning did at the R1 stage of growth. All the interactions were significant, particularly on Lukanga which recorded the highest number of grains per pod at the density of 600 plants ha^{-1}. Lukanga was further affected significantly at all levels of thinning. The interaction of density with thinning (D x T) was significant at 500 K plants ha^{-1} during V2 and R1 thinning stages and at 600 K plants ha^{-1} which was thinned at the R1 growth stage.

Number of pods per plant

Mwembeshi had significantly more pods per plant (16.35), compared to Lukanga (12.24) and SC Semeki (13.73) as shown in Table 2. However, all the interactions on the number of pods per plant were significant (Table 3), particularly for Mwembeshi with density 600 K plants ha^{-1} (16.96) and 700 K plants ha^{-1} (16.55) and for Mwembeshi with thinning times V0 (17.23) and R1 (17.05). Density had no significant influence on pod number except when the 600 K plants ha^{-1} were thinned at R1 (16.85). In terms of the variety x density x thinning time interactions, Mwembeshi, showed that the number of pods increased when thinning was delayed at lower density. At higher densities, however, delayed thinning reduced the number of pods per plant (Figure 4).

Grain weight

Plant density exhibited significant effects only on single effects on grain weight and the highest was observed in 700 K plants ha^{-1} (13.55 g) and the least in 500 K plants ha^{-1} (13.29 g) (Table 2). In the density by thinning interaction, the highest grain weight was recorded in the 700 K plants ha^{-1} at R8 thinning stage combination and the least was in 500 K plants ha^{-1} by R1 thinning time. Mwembeshi at density 600 K plants ha^{-1} thinned at R1 (15.07 g) and exhibited the highest grain weight. The lowest was observed in the interaction of SC Semeki at density 500 K plants ha^{-1} when it was thinned at R1 (12.28 g) (Table 3).

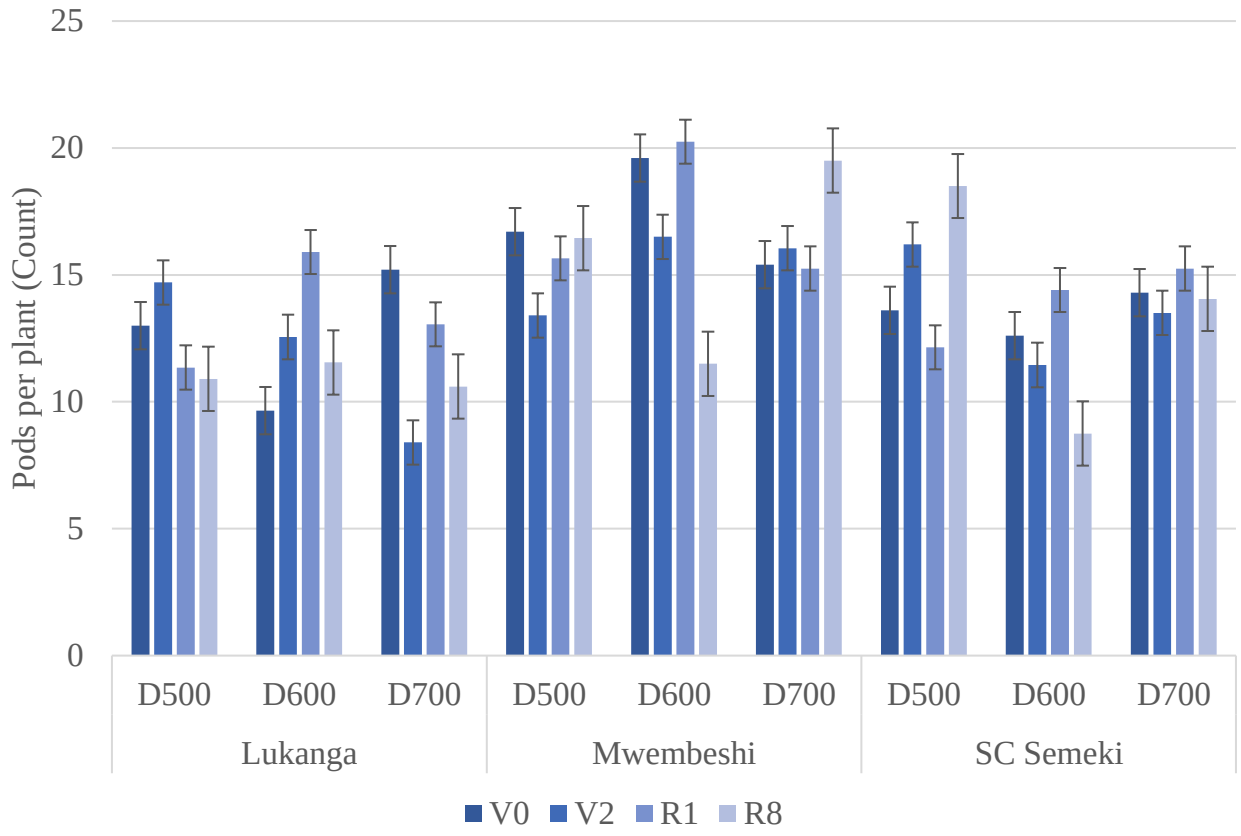

Figure 2. Number of pods per plant of different soybean varieties grown under various plant densities and thinned at different phenological stages (that is, V0, V2, R1 and R8). Vertical bars are standard errors.

Grain yield

Grain yield varied from 1.17 tons ha^{-1} exhibited by SC Semeki to 2.43 tons ha^{-1} exhibited by Lukanga. Mwembeshi yielded 1.95 tons ha^{-1}, which was significantly different from the other two varieties. Across density, thinning at V0 yielded the highest (2.02 tons ha^{-1}) grains (Table 2). Lukanga had significantly higher yield than the other varieties at all density levels. Lukanga also showed the highest yield (2.68 tons ha^{-1}) when thinned at the R1 growth stage. SC Semeki scored the least yield (0.74 tons ha^{1}) at when it was thinned at the V0 phenological stage. The 600 K plants ha^{-1} density exhibited the highest yield (2.51 tons ha^{-1}) when it was thinned at R1. For the density x thinning interaction, the lowest yield (1.42 tons ha^{-1}) was observed at the 700 K plants ha^{-1}, thinned at V0 (Table 3). In the variety x density x thinning time interaction (Figure 5), the highest overall yield (2.92 tons ha^{-1}) was recorded in Mwembeshi at 600 K plants ha^{-1} when thinned at R1. Unthinned treatments (at R8) resulted in least grain yield, 1.76- and 1.54- tons ha^{-1} for Lukanga (at 600 K plants ha^{-1}) and Mwembeshi (at 500 K plants ha^{-1}), respectively. The overall lowest yield (0.62 tons ha^{-1}) was recorded in SC Semeki at 500 K plants ha^{-1} when thinned at the R1 growth stage.

Harvest index

Lukanga had the highest harvest index (0.379) and it was significantly different from SC Semeki (0.345) but not from Mwembeshi (0.352) as demonstrated in Table 2. The 500 K plants ha^{-1} density had the highest HI (0.365) which was significantly different from 700 K plants ha^{-1} (0.352) but not from 600 K plants ha^{-1} (0.361). Thinning at R1 resulted in the highest HI (0.37) and the least was recorded in treatments thinned at R8 (0.35). Lukanga recorded the highest HI when it interacted with density and thinning time at 600 K plants ha^{-1} (0.395) and V0 (0.396), respectively. The highest density by thinning interaction HI was achieved at 500 K plants ha^{-1} thinned at V0. Lukanga had the highest HI at 600 K plants ha^{-1} when thinned at R1. There were tendencies for lower densities to increase HI when thinned early. Delayed thinning resulted to reduction in the HI particularly for Lukanga and SC Semeki varieties (Table 3).

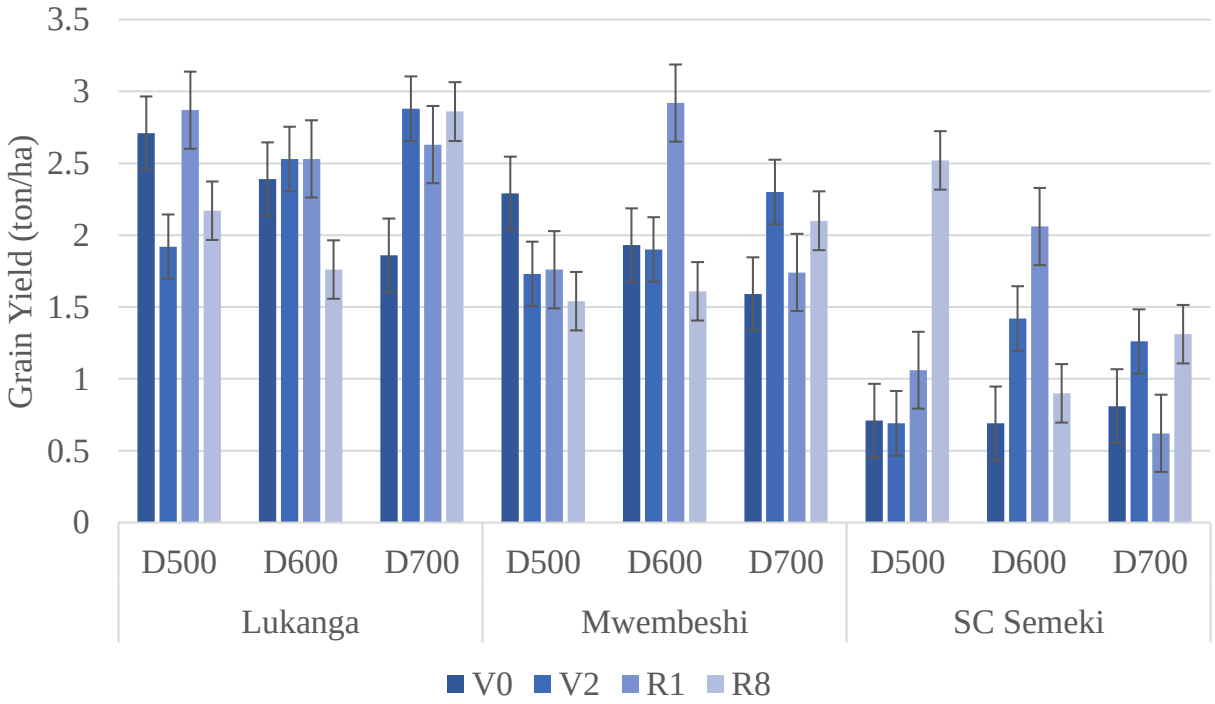

Figure 5. Grain yield of different soybean varieties grown under various plant densities and thinned at different phenological stages (that is, V0, V2, R1 and R8). Vertical bars are standard errors.

Table 4. Correlation of different morpho-physiological and yield parameters.

No.	Parameter	1	2	3	4	5	6	7	8	9	10
1	Plant height at R1 (cm)	-									
2	Plant height at R8 (cm)	0.5946*	-								
3	Biomass (ton. ha^{-1}) R1	-0.0558	-0.1513	-							
4	Biomass (ton. ha^{-1}) R8	0.4402*	0.5275*	-0.1522	-						
5	Root: Shoot ratio	0.0146	0.1065	0.0107	0.0256	-					
6	Grains per pod	0.2855	0.3015	-0.0586	0.2235	-0.0811	-				
7	Pods per plant	0.3078	0.5568*	-0.067	0.4207*	0.0621	0.1701	-			
8	100-Grain weight (g)	0.0001	0.1648	-0.129	0.1729	-0.0198	-0.089	0.1176	-		
9	Grain yield (ton. ha^{-1})	0.3819	0.3853	-0.2379	0.4372*	-0.0294	0.2487	0.1224	0.0874	-	
10	Harvest index	0.1203	0.0004	0.0671	-0.0393	-0.0287	0.5305*	-0.0305	-0.8808***	0.0508	-

Factor significance; *** highly significant ($p \leq 0.001$); **very significant ($p \leq 0.01$); * significant ($p \leq 0.05$) and ns - non-significant ($p > 0.05$).

Correlation

Table 4 shows that plant height at R8 was moderately positively correlated (r = 0.59*) to plant height at R1 (cm) parameters. Biomass at R8 (r = 0.44) and grain yield (r = 0.38) were weakly but positively correlated to plant height at R1. Plant height at R8 had a moderately positive correlation to BM at R8 (r = 0.53*) and to the number of pods per plant (r = 0.55*). Grain yield was weakly positively correlated to BM at R8 (r = 0.43). There was a moderately positive correlation (r = 0.53*) between the number of grains per pod and HI. HI was strongly but negatively correlated (r = -0.88***) to grain weight.

DISCUSSION

The environment under which organisms develop has

influence on resulting morphological and physiological characteristics. Due to their sessile nature, plants cannot move and have to contend with limitations of their environment or stress *in situ*. Thus, to survive and remain productive, appropriate functional plasticity responses are inevitable (Bradshaw, 1965; Takahashi and Shinozaki, 2019). Using various signaling pathways such as Reactive Oxygen Species (ROS), phytochromes and an interaction of physiological metabolites with phytohormones at subcellular level, plants invoke morphophysiological plasticity that builds their competitive effectiveness at resource mobilization (Bradshaw, 1965; Wani et al., 2016; Mhamdi and Van Breusegem, 2018; Takahashi and Shinozaki, 2019; Wahid et al., 2007). Plant density is one of the most pliable factors that can be altered in the row crop growth environment to improve crop production (Wright and Lenssen, 2013).

Effect on vegetative parameters

Variety had significant effects on all vegetative parameters except on root to shoot ratio. Lukanga had the highest plant height (at R1 and R8) and biomass (R8), which was significantly different from the other two. Contrasting performance of Lukanga and SC Semeki despite both being determinate varieties was also found by Mataa and Sichilima (2019) cannot be explained only on the basis of growth characteristics but other genetic parameters (Tekola et al., 2018).

SC Semeki accumulated higher biomass during the vegetative phase than in the reproductive phase. Biomass and root-shoot ratio are important indicators of plant vigour in an environment where interplant competition exists (Yang et al., 2019). Plants with higher biomass accumulation and comparative root mass are more competitive in accessing nutrients and moisture (Craine and Dybzinski, 2013). The reduction in biomass accumulation we observed for SC Semeki in comparison to other varieties could be attributed to vegetative allocational plasticity (Mataa and Sichilima, 2019), an important trait in resource competition (Sultan, 2003). Similar effects have also been observed in perennial plants during the immature phase (Mataa and Tominaga, 1998a). It can, therefore, be suggested that the value of SC Semeki is more as a vegetative or forage use.

Planting density showed no significant effects except on root: shoot ratio. Our findings on plant height were similar to the observation made by Mellendorf (2011), who associated the slight decrements/increments between densities to general plant growth due to row spacing and to the decreased/increased access to photosynthetically active radiation (Mataa and Tominaga, 1998b; Park and Runkle, 2018). A similar study by Sichilima et al. (2018) showed similar findings, where density had no significant effects on the plants.

Maintaining the crop without thinning up to R8 appeared to increase root to shoot ratio. The increased root biomass in relation to above-ground biomass can be attributed to the sustained stress. Plants invoke photoassimilates allocational plasticity (Murren et al., 2015) when under stress which increases photoassimilates partitioning to roots hence increasing root surface area to forage and competing for resources in the root zone (Rondanini et al., 2017).

The findings on three-way interactions suggested that for Lukanga, plant height was stable across planting densities and thinning times. Mwembeshi had lowest plant height at the 500 K density. Thereafter, it increased to reach a maximum at 600 K and fell at 700 K. SC Semeki showed a similar trend, but it was true only for V0 and V2. Generally, delaying thinning reduced plant height for Lukanga but did not for Mwembeshi and SC Semeki. Biomass decreased with planting density in Lukanga, was stable for SC Semeki across plant densities, whereas Mwembeshi was intermediate. In all, genotypes delaying thinning reduced biomass.

Effect on reproductive parameters

The number of grains per pod, number of pods per plant and grain weight are important yield components (Pereira-Flores and Justino, 2019). Lukanga had a significantly higher number of grains per pod while Mwembeshi had higher number of pods per plant contributing to significantly higher grain yields. Mwembeshi, an indeterminate variety responded by developing more pod than increasing grain weight. Rondanini et al. (2017) observed reproductive plasticity in spring rapeseed to be related to floral branching. Based on our results, we postulate that Lukanga, a determinate variety exhibited reproductive plasticity mainly due to the changes in the number of grains per pod. Mwembeshi, on the other hand, exhibited reproductive plasticity related to the number of pods per plant. The response in Mwembeshi is in conformity with the established theory that modular organisms have compensatory capabilities to any adverse shocks faced during plant growth (Murren et al., 2015). Agudamu et al. (2016) concluded that modular plants have a longer period over which they have to adjust to environmental stress through branch, leaf area or pod number development.

Thinning early exerted significant effects on grain yield and harvest index. Relieving the imposed density stress after the plants have fully enabled development of competitive capacities for environmental resources in the early phenological stages and allows the plants to concentrate on reproductive structures such as number of pods per plant (Mellendorf, 2011).

Plant density had little or no effect on reproductive and vegetative parameters. However, lower planting densities

increased HI when thinning was done early. Similar results were observed by Shamsi and Kobraee (2011) who revealed that HI declined with an increase in plant density. Mellendorf (2011) made similar observations that large differences were apparent in the high plant density, where cohorts relieved of competition at V3 increased HI by 7.8% compared to competition relief at R4 where HI was reduced by 10%.

The three way interactions showed that grain yield was stable across plant density and thinning stages for Lukanga and Mwembeshi but it fell with increase in plant density for SC Semeki. SC Semeki showed slight increases in grain yield with plant density up to 600 K density then it declined again at 700 K density. Thus, SC Semeki had comparatively low yield at each thinning stage compared to the other two genotypes.

The positive, though weak correlation between grain yield and biomass weight (r = 0.43) suggested that larger plants tend to have higher yield. Mellendorf (2011) noted that treatments that had high biomass were also reported to have high number of branches, high number of pods per plant and higher grain yield. A close relationship between grain yield and biomass at R8 was shown by the regression analysis. Similar findings were obtained by Duncan (1986) who associated higher biomass to higher grain yields. Sichilima et al. (2018) also concluded that biomass had the strongest effect on yield. The results suggest that plant phenotypic responses to the environment can be wider than alternative and unchangeable pathways suggested by current plasticity concepts (Grime, 1977; Sultan, 1992 and Sultan and Bazzaz, 1993).

Conclusion

Lukanga and SC Semeki are both determinate varieties but seem to follow different pathways in their responses to density stress. The lack of effect of planting density exemplified by yield being constant at different plant density (or failure to increase yield at high planting density) suggests that maintaining a low seed rate is more economical given the high cost of seed. Our results, especially with reference to Mwembeshi, suggest that in addition to typical plastic responses, soybeans can show non-plastic or ‘elastic responses’ allowing the plant to recover after stress is removed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Sub-acute and chronic toxicity of silver nanoparticles synthesized by *Azadirachta indica* extract

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In this study, biosynthesis of silver nanoparticles (AgNPs) and its toxicity were investigated. Different functional groups responsible for adsorption, morphology and absorption of the nanoparticle were characterized using UV-Vis, Fourier transmission infrared spectroscopy (FTIR), and scanning electron microscope (SEM) analyses, respectively. The toxicity of orally administered *Azadirachta indica* AgNPs was assessed in Swiss albino rats. Sub-acute toxicity was determined in daily dosages from 30-0.3 mg/kg body weight for 28 days. Chronic toxicity was determined in two dosages 30 and 10 mg/kg body weight for 180 days. Control groups were included and were administered with distilled water. The UV-Vis spectroscopy showed surface plasmon resonance of 430 nm for the silver nanoparticle. The FTIR spectrum showed primary-N-H bond and secondary-C-N amides. The nanoparticles synthesized were 45 nm average sizes. There were no significant differences (P>0.05) observed between the packed cell volume (PCV), animals body weight of the control and treatment groups in sub-acute and chronic toxicity. Portal hepatitis in the liver at the 30 mg/kg b. wt was noted. Hence, histopathology examinations confirmed the liver damage noted in clinical biochemistry. Kidneys histological organization appeared normal generally with glomeruli and tubules visible. Our results demonstrate that *A. indica* silver nanoformulation may be safe at daily dosage of up-to 10 mg/kg b. wt. However, it indicates that the *A. indica* silver nanoformulation on daily use at 30 mg/kg may lead to liver damage.

Key words: Silver nanoparticles, toxicity, *Azadirachta indica,* rats.

INTRODUCTION

Therapeutic activities of neem plant (*Azadirachta indica*), a member of the Meliaceae family, originally found in India, Pakistan, Bangladesh, and Nepal (Abdelhady et al., 2015). Several activities from neem have been reported including antibacterial, antifungal, and anti-inflammatory (Alzohairy, 2016). *A. indica* extracts have

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been encapsulated in various nanoparticles (NPs) such as Silver (Ag), Gold (Au) and chitosan Dash et al., 2017). Silver nanoparticles (AgNP) is one of the most used nanoparticles in drug formulation due to its potent broad-spectrum of antibacterial properties, strong permeability and little drug resistance (Wen, 2017). Methods like nano clustered lipid carrier appears to be optionable for delivery to overcome disadvantage of lower loading capacity and drug expulsion in storage Elsaesser and Howard, (2012) however, in recent studies they showed moderate toxicity of elevated alkaline phosphatase (ALP) (Okeahialam et al., 2000). Biosynthesis of silver nanoparticles have been claimed to support optimal green chemistry metrics (Bilal et al., 2017). The green synthesis methods have proven to be consistent, economic and biocompatible, environmental and eco-friendly (Luo et al., 2018). Plant-mediated green chemistry approach indicates numerous advantages, including a significant yield production at a minimal cost (Singh et al., 2016). The study by Nwagbogu (2018) depicted that the ethanolic *A. indica* bark extract had antiproliferative activity against HCT116 human colon cancer cell, MCF7 breast cancer cells and Hep-G2 human liver cell line. In that study, encapsulation of *A. indica* bark extracts with silver nitrate increased its *in vivo* tumor growth inhibitory activities in Swiss albino rats (a popular mammalian model), showing a 71. 96% inhibition capability at the dose of 30 mg/kg b.wt. The lethal dose (LD50) of the *A. indica* AgNPs was found to be higher than 160 mg/kg and no organ damage was noted after daily administration for 14 days. This study further determined the sub-acute (28 days) and Chronic (180 days) toxicity effect after administration of *A. indica* silver nanoparticles (NPs) in Swiss albino rats.

MATERIALS AND METHODS

Collection and plant material authentication

The bark of *A. indica* plant was collected in December 2018 from Kiambu county, Kenya. It was authenticated by Mr. John Kamau Muchuku, Department of Botany, Jomo Kenyatta University of Agriculture and Technology (JKUAT). A voucher specimen (ET001ABC) was deposited in the University Herbarium.

Methanolic extraction

The bark specimen of *A. indica* were washed with distilled water, air-dried inside a greenhouse for two weeks and pulverized into coarse powder using grinding machine. The coarse powder was further processed to fine particles by sieving. The powdered bark (50 g) was macerated in 70% Methanol. The mixture was allowed to stand for 72 h with vigorous shaking. Following the method described by Nwagbogu (2018), methanol extract was then filtered using Whatman' filter paper No. 1 and the filtrate was concentrated under pressure at 45°C in a rotary evaporator to remove methanol and the remaining water. The extract was freeze dried using a freeze dryer (mrc # Israel). The resultant extract was weighed, labelled and stored in an airtight container at -20°C prior to use.

Preparation of AgNPs

One millimole of silver nitrate ($AgNO_3$) was weighed, bark extract prepared and used for synthesis of silver nanoparticles. Methanolic extract of the bark of *A. indica* was added to silver nitrate solution in a ratio of 4:1 for bio-reduction process at room temperature (25°C) following the method discussed by Abdullah et ai. (2016) in his study. The progress of the reaction between silver metal ions and the bark extract was monitored by performing periodic sampling (at 3 h intervals for 12 h) using a UV spectroscopy. The Nano-formulated product was purified by centrifugation at 5000 rpm for 5 min. The supernatant was discarded and then the precipitated silver nanoparticles were washed with deionized water 10 times for complete purification. The resultant product was dried in vacuum chamber for 24 h at 35°C to obtain the dry silver nanoparticles (Neran and Lamia, 2016).

Classification and characterization of *A. indica* nanoparticles

The synthesized nanoparticles were subjected to UV- Spectroscopy and Fourier-transform infrared spectroscopy (FTIR) to confirm the successful synthesis of silver nanoparticles and detect the key functional groups present in the extract, which are responsible for capping of silver nanoparticles, respectively. Scanning electron microscope (SEM) was performed for Scanning electron morphological analysis of Silver nanoparticles following the method described by Lalitha et al., (2013).

Animals

Male Swiss albino rats (*Mus musculus*), 8-12 weeks old weighing between 180 and 200 g were bred in the animal house at JICA SAFARI House, Jomo Kenyatta University of Agriculture Technology Kenya. The animals were kept in clean plastic cages placed in a well-ventilated house with optimum condition (temperature: 28 ± 2°C; photoperiod: 12 h natural light and 12 h dark; humidity: 40-45%). They had free access to commercial pelleted rat feed and water *ad libitum*. The floors of the cages were filled with paper cuts while the cage cleaning was done on daily basis.

Administration of synthesized nanoparticles to swiss albino rats

Animals (Male rats) were divided into six groups for sub-acute toxicity study based on the dosage administered Group 1: (30 mg/kg), Group 2 (10 mg/kg), Group 3 (3 mg/kg), Group 4 (1 mg/kg), Group 5 (0.3 mg/kg) of nano-formulation and Group 6: negative control (distilled water). For chronic toxicity study, the animals were divided into three groups based on the dosage administered group1: (30 mg/kg), Group 2 (10 mg/kg) of nano-formulation and group 3: negative control (distilled water). 0.2 ml of the drug was orally administered once daily for 28 and 180 days for sub-acute and chronic toxicity test respectively. Rats were fasted overnight prior to dosing. Following the period of fasting, the animals were weighed and then the test substance was administered orally using 5 ml oral gavage.

Clinical signs and measurement of body weight

Observations were made before and 3 h after dosing and they were recorded as per the guidelines provided by Organization for Economic Co-operation and Development (OECD, 2002). They included any change in appearance behavior, hair, feces and

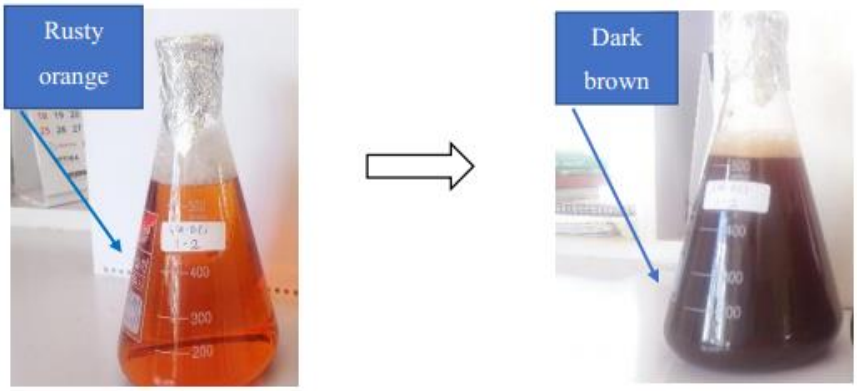

Figure 1. (A) *A. indica* bark extract + $AgNO_3$ at time T=0 min (Rusty-orange colour) (B) *A. indica* bark + $AgNO_3$ at time T = 72 h (Dark brown colour).

mortality. Changes in weight, an important toxicity index for rats, were measured before grouping and dosing.

Sample collection

On the last day and after an overnight fast of 8 h, the animals were sacrificed under carbon dioxide asphyxiation. Blood samples were collected by cardiac puncture and collected into clean bottles for hematological and biochemical investigations. The liver and kidneys were excised from dissected rats, after which they were washed using physiological saline and weighed. The liver and kidneys were then fixed in 10% formalin saline for further histopathological processing. Tissues were embedded in paraffin wax and sections of 3 micron were prepared and stained with hematoxylin and eosin according to the procedure described by Slaoui and Fiette (2011).

Packed cell volume determination (PCV)

The PCV was determined using the micro hematocrit method (Shamaki et al., 2014). Briefly, an aliquot of blood sample with anticoagulant from each rat was placed in micro-capillary tubes and then centrifuged at 14000 rpm for 10 min. Samples were analyzed for PCV after centrifugation, using a micro-capillary reader.

Biochemical tests

The sub-acute toxicity assay involved, the levels of Aspartate aminotransferases (AST), Alanine aminotransferases (ALT), Urea, and creatinine levels were analyzed using standard diagnostic test (Point of care, Roche: Refrotron test strips) kits on Automated Clinical Biochemistry analyser (Reflotron Plus System®, model: Cobas 4800 Detection Analyzer; India).

Gross pathology and histopathology

Tissues harvesting

Signs of hemorrhages, ecchymosis and petechial appearances were assessed after 28 days and 180 days of drug administration. Tissue specimen from the liver and kidney were collected and preserved in 10% buffered formalin. Thereafter, the organs were processed for histopathological examination following the method as described by Ingelheim (2003), after 180 days of *A. indica* silver nanoformulation treatment.

Statistical analysis

Data collected from the biochemical and hematological analysis were expressed as mean ± SEM. One-way ANOVA and column statistics was used to compare means of treated and control rats for the different parameters assessed. The significance level was set at $P \leq 0.05$.

Ethical approval

Approval for animal experiments was granted by the Jomo Kenyatta University of Agriculture and Technology Animal Ethics Committee. The protocols were approved by the Institutional Animal Care and Use Committee (IACUC) REF: JKU/2/4/896B at JKUAT and conducted in compliance with Kenya's national ethical standards to minimize the suffering of animals.

RESULTS

Characterization of AgNPs

UV Vis spectroscopy

The prepared *A. indica* nano-formulated extracts were isolated to different particle sizes using size fractionation process by a centrifugation at different intervals. The specific speeds and uniform sizes were selected for the study. The UV–Vis spectrum illustrated in Figure 2, depicts a well-defined absorption peaks for the particle sizes. These correspond to the wavelength (500 nm) of the surface plasmon resonance (SPR) of *A. indica* nano-formulated extracts. Colour change from rusty orange at time 0 to dark brown at 72 h and peak formation at about 400-480 nm confirmed bio-reduction of silver ions by the extract and the successful nanoformulation (Figure 1).

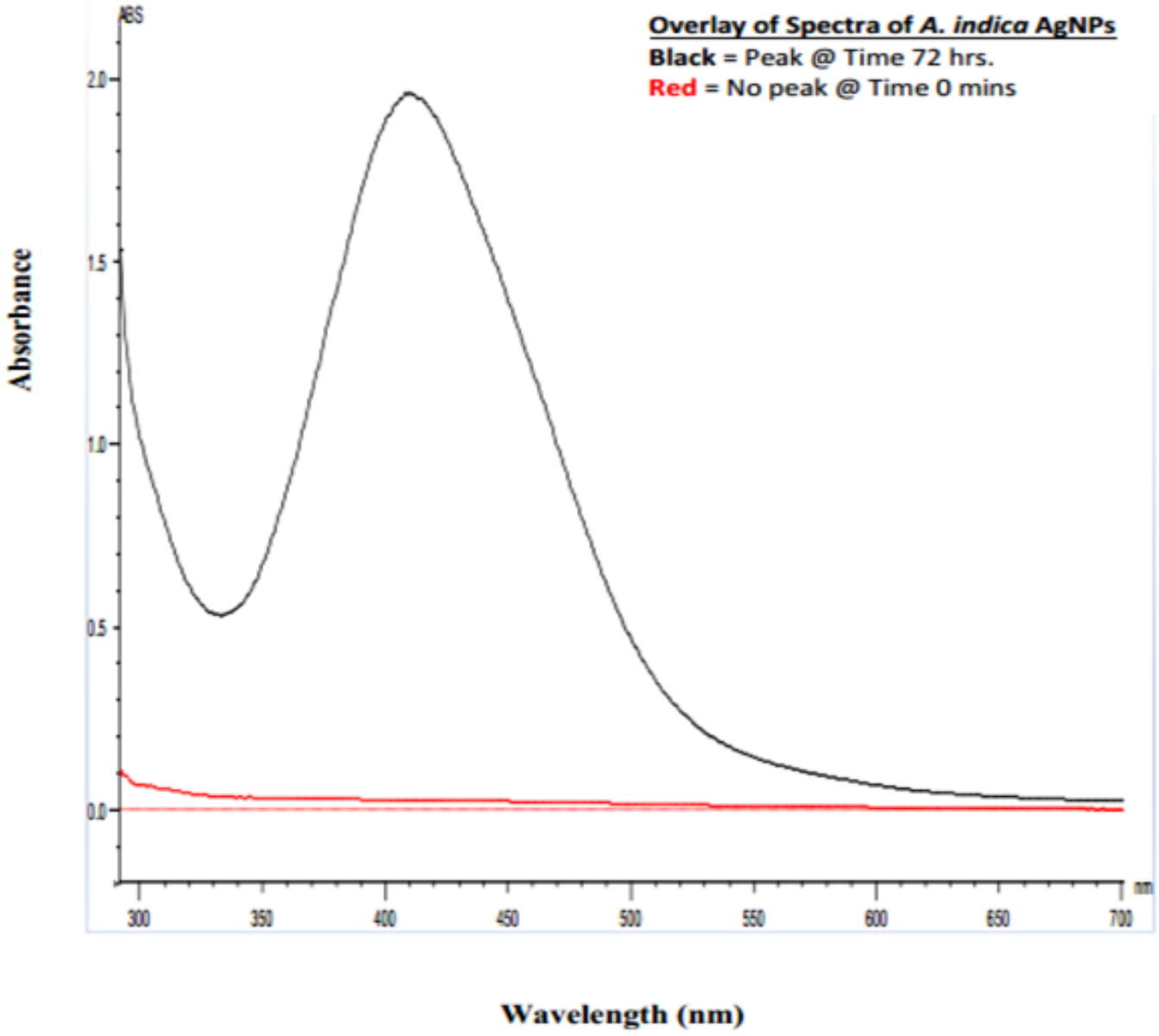

Figure 2. UV/Vis absorption spectrums of silver ions reduction (red colour line) to AgNPs (black colour line), the spectra in the low and high wavelength regions were included. The absorption spectrum was recorded at room temperature after 72 h of the preparation period.

Fourier transform infrared spectrometer (FTIR)

The transmittance bands of different functional groups varying between 4000 and 500 cm^{-1} was recorded for the FTIR spectra, which allows for high reactivity, specificity and compound solubility in water (Coates, 2004) (Figures 3 and 4). The results depict the presence of hydroxyl /alcohol groups at 3679.9 cm^{-1}, alkyne C-H, saturated aliphatic groups of alkane and hydrocarbons including methyl C-H at 2935.5 cm^{-1} and methylene at C-H 1595.0 cm^{-1}, aliphatic fluoro compounds at 1143.7 cm^{-1} and aliphatic phosphates at 1112.9 cm^{-1}. This was compared to the study of Tensingh (2017) and Amuanyena et al. (2019).

Scanning electron microscopy (SEM) analysis

The result shows the high-density Ag nanoparticles synthesized by *A. indica* bark extracts and further confirmed the presence of silver nanoparticles. It was shown to be relatively spherical in shape and polydisperse with 10 µm width (Figure 5a). As portrayed in the micrographs, *A. indica* silver nanoparticle appears to have

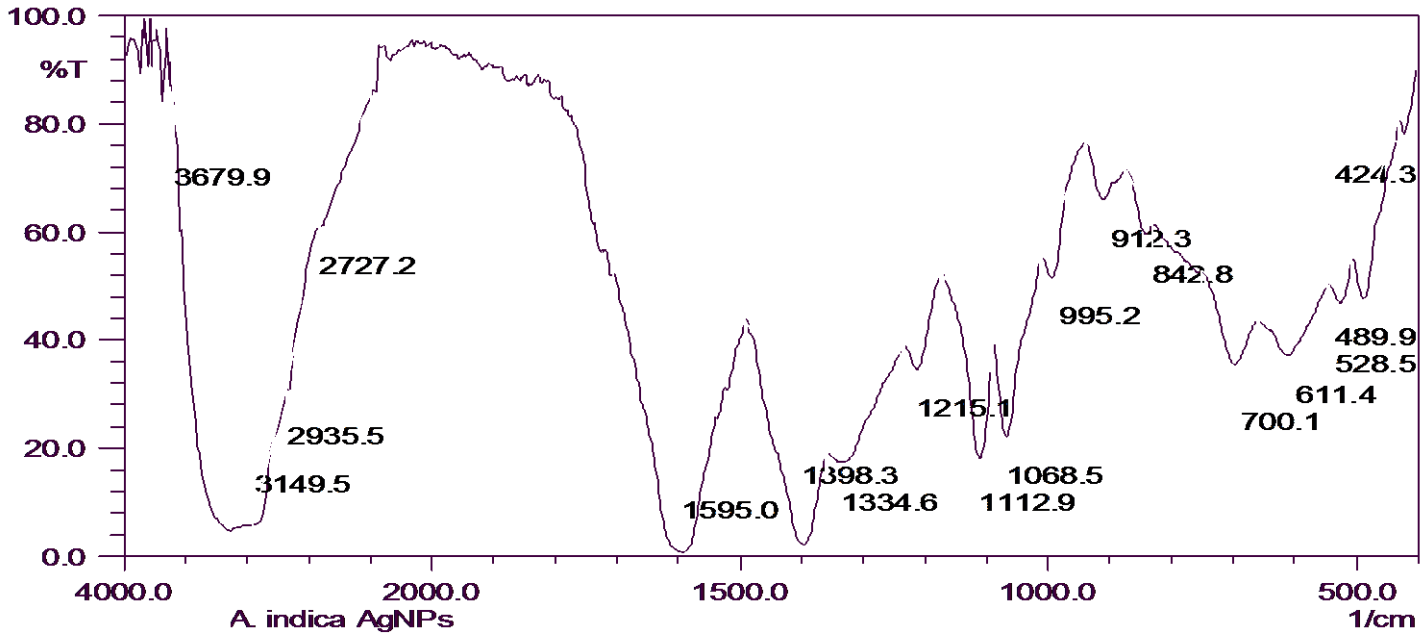

Figure 3. Shows the FTIR absorbance spectra of the *A. indica* silver nano-formulation.

an energy dispersive spectrometers (EDS) pattern which indicates that 13% presence of Ag ion (Figure 6). The EDS spectrum also reveals (C) peak (Figure 6).

Clinical observations and body weight measurement for 28 and 180 days

Clinical

The administration of dose up to 30 mg/kg of *A. indica* nano-formulation did not lead to mortality. No clinical signs of toxicity were recorded during the 28 and 180-days experimental period of all doses of *A. indica* silver nanoformulation. The skin, fur, water intake, food intake, mucous membrane, and urination of rats were found to be normal after treatment. Additionally, diarrhea, fast breathing, lethargy, inactivity, liquid secretions from eyes or excessive salivation were not observed in both sub-acute and chronic toxicity study.

Body weight

The body weight of control non-treated group varied between 144 to 394 g at the beginning and end of the treatment period, respectively. Following treatment, no significant variations ($p>0.05$) of body weight was observed between the control groups and the *A. indica* nanoformulation treatment groups for both sub-acute and chronic toxicity study.

Packed cell volume (PCV) for both sub-acute and chronic toxicity

PCV levels of control groups ranged between 37-50%, the values were within the normal range. There were no significant differences ($P>0.05$) between the control and the treatment groups in sub-acute and chronic toxicity study.

Biochemical analysis

Hepatic: The serum ALT levels of untreated control groups ranged from 102 to 130 µ/L. There were no significant differences ($P>0.05$) in serum ALT levels between the untreated control group and the treatment groups below 10 mg/kg after 28 days treatment period. However, there was a significant increase ($P<0.05$) in ALT of 30 mg/kg treatment groups. In addition, at 30 mg/kg b. wt, there were significant increase ($P<0.05$) in ALT after 180 days treatment period. The serum AST levels of untreated control groups ranged from 135 to 188 µ/L. There was significant increase ($P<0.05$) in AST of 10 and 30 mg/kg after 28 days treatment period.

Kidney: The blood urea levels of untreated control groups ranged from 5 to 8.88 mmol/L. There were no significant differences in blood urea levels ($P>0.05$) between the untreated control group and the treatment groups in sub-acute and chronic toxicity experiment. The blood creatinine levels of untreated control groups ranged from 44.20 to 57.7 µmol/L. There were no significant

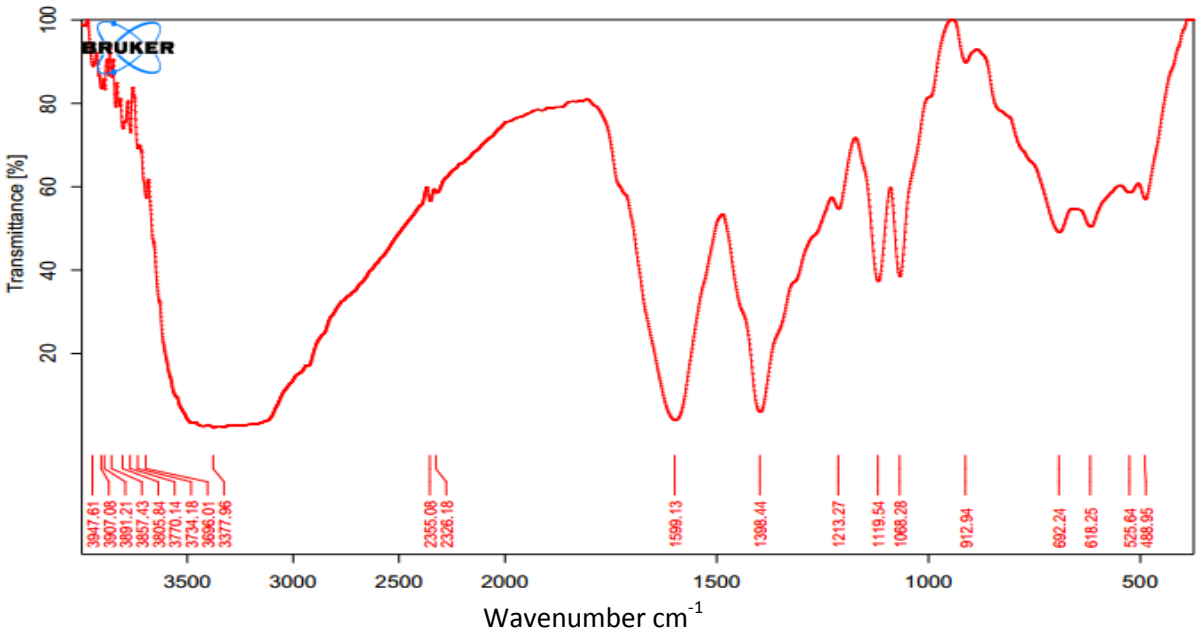

Figure 4. FTIR transmittance (%) spectra of the *A. indica* silver nano-formulation.

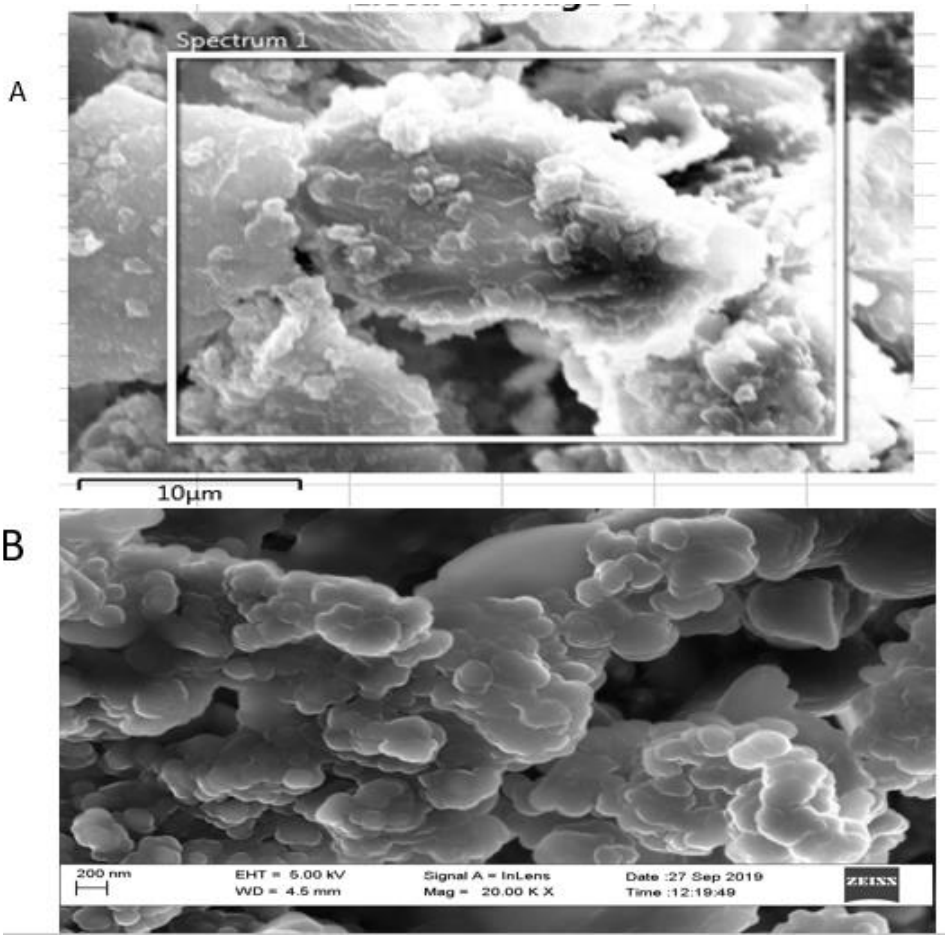

Figure 5. SEM image of dispersed, spherical shaped *A. indica* Ag nanoparticles.

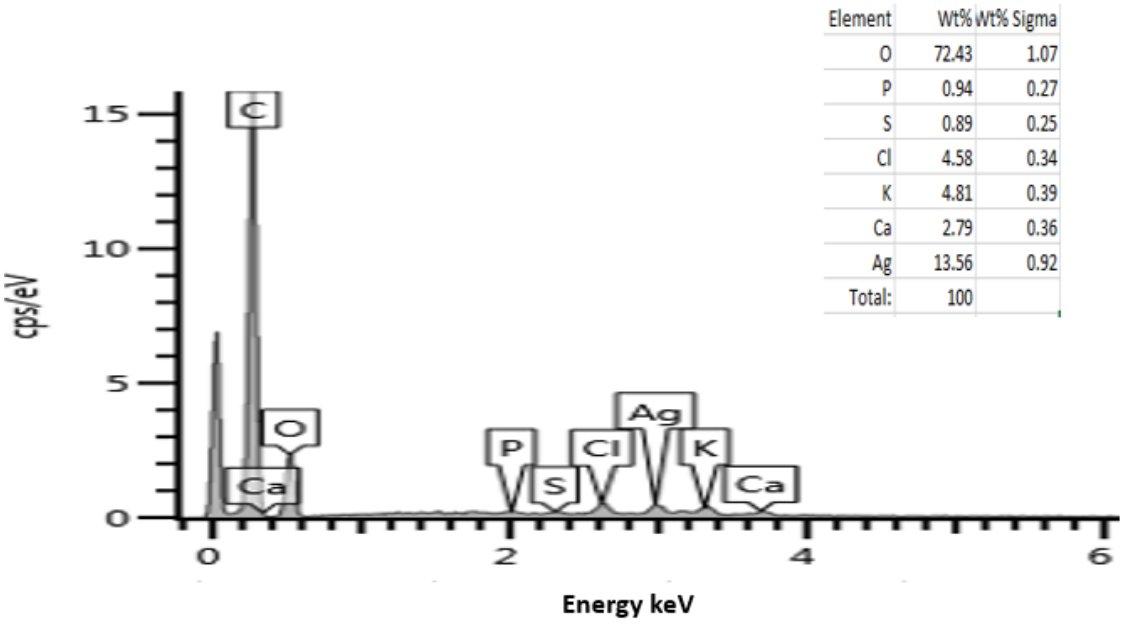

Figure 6. Shows the spectrum peak element in kiloelectron volt (KeV) and their percentage.

Table 1. Biochemical profiles of Swiss albino rats administered with silver nanoformulated *A. indica* after 28 and 180 days of treatment.

Dose (mg/kg)	Sub-acute/chronic toxicity (days)	AST(U/L)	ALT(U/L)	Urea (mmol/L)	Creatinine (μmol/L)
30	28	300.5±11.5**	152±3.04**	9.275±0.92	46.6±0.5
	180	ND	168.3±6.69*	6.767±0.32	46.77±1.3
10	28	289±14**	123.5±11.5	8.825±0.80	44.85±0.65
	180	ND	153.3±6.76	6.6±0.20	45.23±1.03
3	28	236±18	119±8	7.705±0.29	44.8±0.4
1	28	215±17	125.5±4.5	8.505±0.89	45.2±1.0
0.3	28	220.5±13.5	111.5±4.5	7.23±0.86	44.7±0.5
Control	28	161.5±26.5	104.5±2.51	7.915±0.96	48.85±1.15
	180	ND	103.2±20.64	5.7±0.51	50.37±3.79

Notes: Values are expressed as mean ± SEM, ND-Not Done, n=3. *P<0.01, **P<0.001 compared to the control. ALT, alanine aminotransferase; AST, aspartate aminotransferase; control, rat treated with distilled water; SEM, standard error of mean.

differences between blood creatinine levels (P>0.05) of the untreated control groups and the treatment groups in both sub-acute and chronic studies (Table 1).

Gross pathology and histopathology after sub-acute and chronic toxicity

Sub-acute toxicity gross and histopathology: The liver of rats administered with *A. indica* silver nanoformulation showed a smooth surface and were evenly dark red colored. Rats administered the dosage of 0.3 and 3 mg/kg showed normal hepatocellular architecture. Rats administered the highest dosage (30 mg/kg) showed signs of portal and interface hepatitis. Kidney appears smooth surfaced and evenly dark red in color. They presented a normal histological structure with a normal appearance of the renal cortex, glomeruli and tubules in the low dose of 0.3 and 3 mg/kg. Kidneys presented a normal ultrastructure but had some congested blood vessels (BVC) in the group administered 30 mg/kg (Figure 7).

Chronic toxicity gross and histopathology: Similarly,

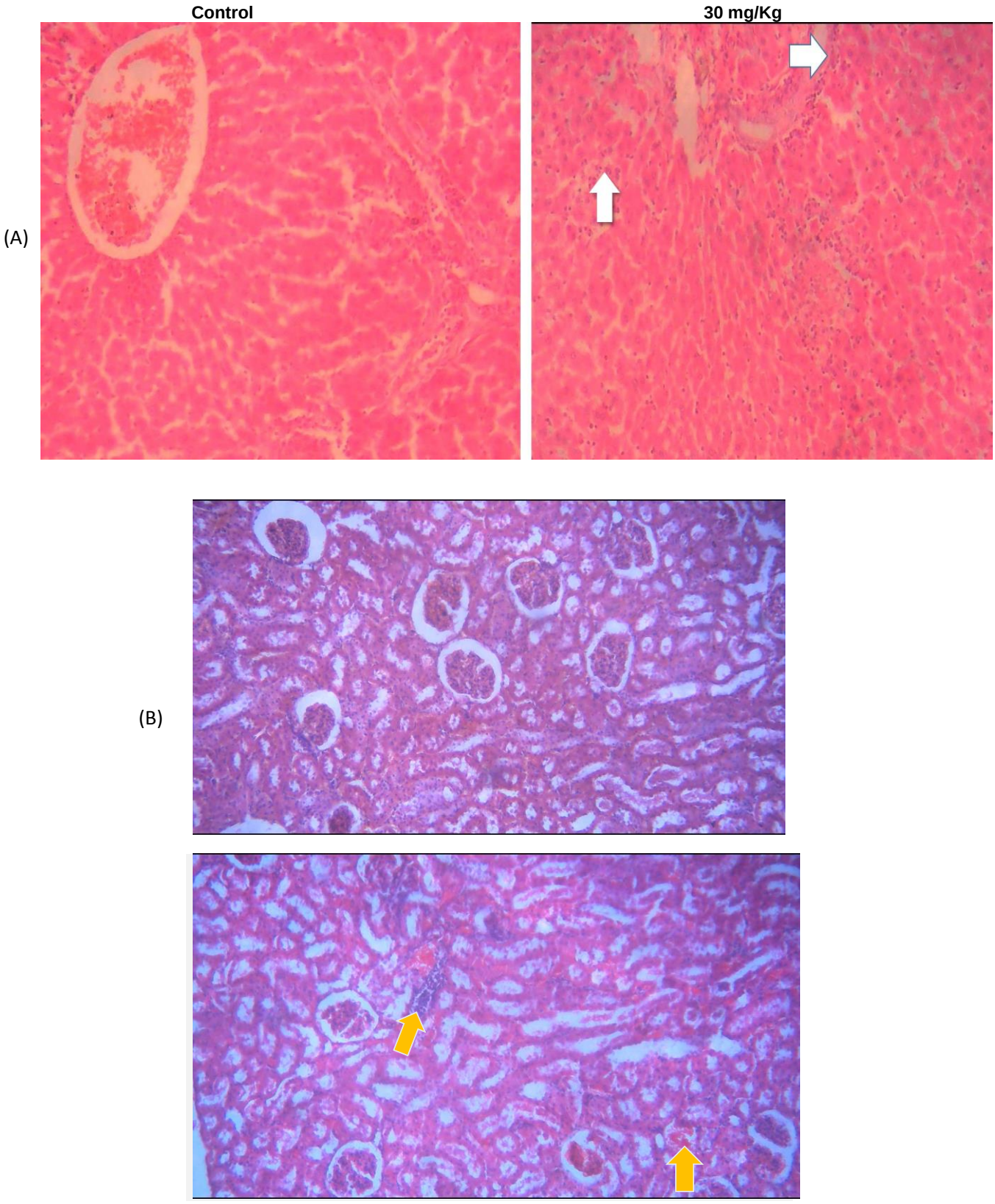

Figure 7. Histopathological examinations of the kidney and liver of rats after 28 days of drug administration. A. Represent liver and B. Kidneys. The arrows make a representation of portal infiltration in the liver treatment group tissues and a presence of congested blood vessels in the kidney treatment group tissues. The sections were stained with hematoxylin and eosin. *A. indica* AgNPs treatment group up to (30 mg/kg), H&E x400.

to the 28 days drug administration effect, the liver of rats administered up to 30 mg/kg showed a smooth surface and evenly dark red colored. Rats administered 10 mg/kg showed normal hepatocellular architecture but with congested blood vessels (CBV). They presented a normal renal architecture with glomeruli and tubules having normal structure. However focal zones of hemorrhage (ZH) were observed in the medullary interstitium. The highest dose of 30 mg/kg was associated with periportal infiltration in the liver indicating portal hepatitis and an infiltration in cortex including glomeruli. Kidneys of rats administered up to 30 mg/kg showed a smooth surface and evenly dark red colored. Further kidneys showed a proliferation of podocytes (PD) limiting bowman capsule space and a renal tubular lumen with exudate (Figure 8).

DISCUSSION

The synthesized particle size had a maximum Surface Plasmon Resonance (SPR) peak at 430 nm. Various reports have established that the resonance peak of silver nanoparticles appears around this region, but the exact position depends on a number of factors such as particles size, and the surface-adsorbed species (Wen, 2017). Absence of absorbance at wavelengths greater than 550 nm indicated their well-dispersed state in solution (Coates, 2004). Scanning electron microscopy (SEM) showed that the AgNPs formed were spherical in shape with an average diameter of 10 µm and an average size of 45 nm. Similar results have been reported by Nwagbogu (2018). The electrostatic relations and hydrogen bond between the bio-organic capping molecule bonds are attributed with the synthesis of silver nanoparticles using plant extract, (Heera et al., 2015). The AgNPs were also characterized using EDS spectroscopy. EDS analysis provides qualitative and quantitative status of elements that may be involved in the formation of Silver nanoparticles (Sulaiman et al., 2017). The EDS spectrum was recorded in the profile spot mode as demonstrated in Figure 6. The outcomes clearly illustrate the presence of strong signals from the NPs which confirmed the successful biosynthesis of AgNPs by methanolic bark extract of *A. indica*. Weaker signals from P, Cl, K, Ca and S atoms were also noted. These weak signals may originate from macromolecules like metabolites and fatty acids present in the plant. A peak related to O atom was observed in the EDS spectrum, which is probably origin of the glass substrate or from the SEM chamber (Ismail et al., 2018). The same was reported and was also explained by Wang et al., (2016). Whereas, other reports depicted that O present in EDS analysis may be applied during preparation processes (Li et al., 2012). However, from the EDS spectrums, it is evident that silver particles were reduced by methanolic bark extract of *A. indica* and had the weight percentage of silver of 13.56%, as shown in the inset of Figure 6. The inset table also illustrates the atomic weight percentage of other elements. The biomolecules responsible for reducing Ag+ ions and capping the bio-fabricated AgNPs were presented at different functional groups such as the alkane, alkene, amine, and carboxylic acid. They have been recently reported to be major reducing agents in the biosynthesis of silver nanoparticles (Amuanyena et al., 2019). This in addition plays majorly the role of indicator of the presence of residues of plant phytochemicals in the synthesized silver nanoparticles, (Taha et al., 2019). Therefore, it shows in the FTIR results the NPs synthesized were possibly surrounded by proteins, fatty acids and some metabolites namely terpenoids having functional groups of amines, alcohols, ketones, aldehydes, and carboxylic acids similar to the recent findings of Taha et al. (2019). The presence of metabolites and proteins act as reducing agent and also as stabilizing agent and may avoid accumulation by binding to AgNPs through free amino groups through electrostatic magnetism of negatively charged carboxylate groups in extracellular enzyme filtrate from *A. indica* (Suresh et al., 2011; Ammar and El-Desouky, 2016). Consequently, the findings observed in FTIR spectrum discovered the presence of different biomolecules that acted as reducing and capping agents that were involved in the AgNPs formation and its stability, (Taha et al., 2019).

In this study, no mortality was noted after oral administration of dosages of up to 30 mg/kg of nanoformulation synthesized using methanolic bark extract of *A. indica* in the rats. According to Nwagbogu (2018) neem bark extracts has a low toxicity and its LD_{50} (lethal doses) has been evaluated to be greater than 300 mg/kg in mice. This is the lowest toxicity class and according to the Guidance Document on Toxicity (Sharma, 2010), the *A. indica* silver nanoformulation may be assigned to be class 5 (LD50 > 30 mg/kg body weight). The biochemical parameters such as ALT and AST are among the biomarkers for the liver function. In normal rats the ALT level observed in this study was comparable with the finding by Ong et al., (2016) who showed that the ALT level of Albino mice ranged from 55.0-208.2 µ/L. There was a significant increase (3 times higher) of the ALT at a dosage of 30 mg/kg treatment groups after 28 days and 180 days of drug administration. However, the animals were not further monitored after the experiment. Similar to the ALT, there were significant increase of the AST at 10 and 30 mg/kg treatment group after 28 days of drug administration. Despite our gross pathology results showing evenly dark red and normal smooth surfaced liver, the histopathological findings showed periportal infiltration by inflammatory cells suggesting portal hepatitis. This may be confirming the clinical biochemistry results. It may suggest that the *A. indica* silver nanoformulation on

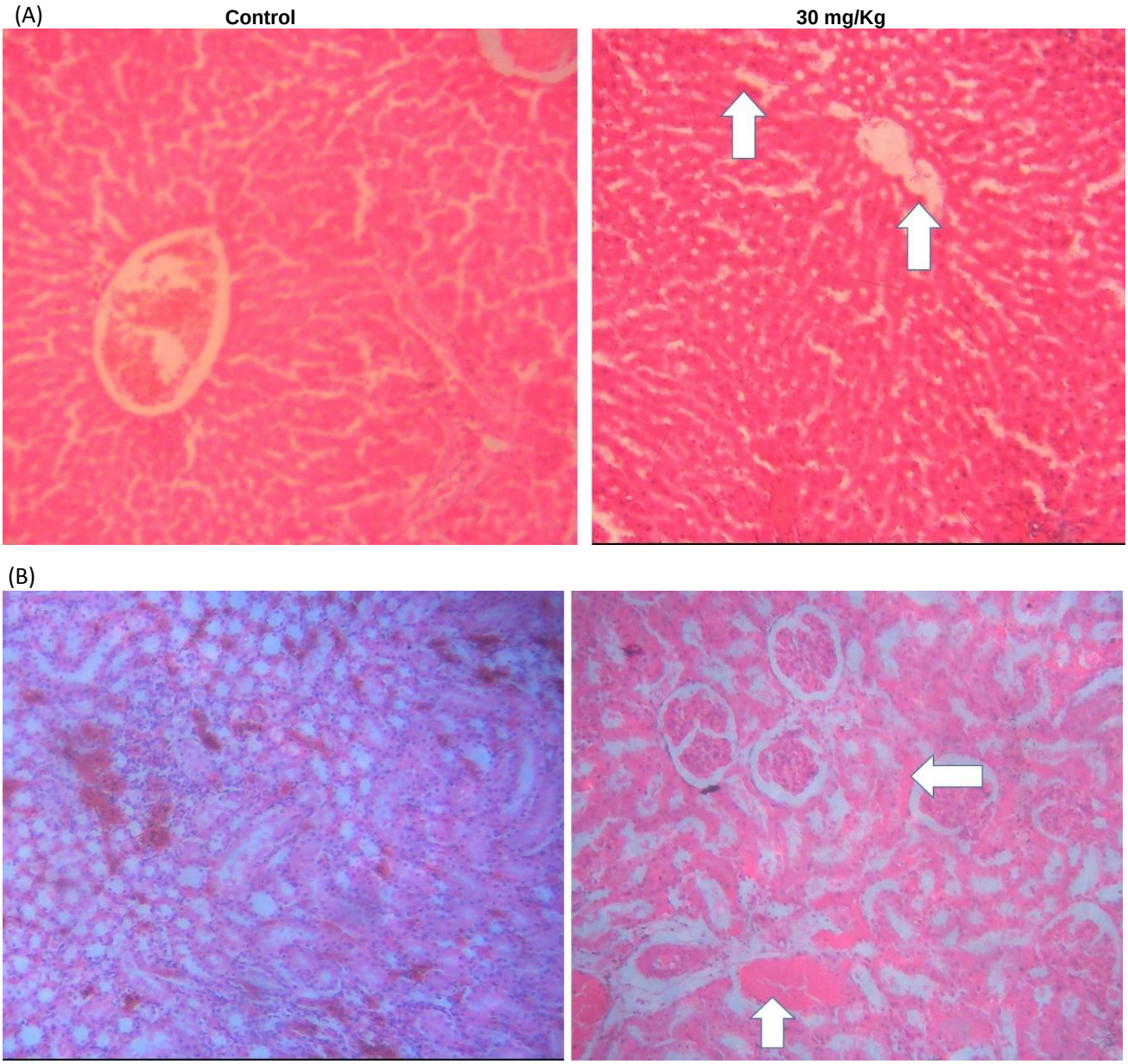

Figure 8. Histopathological examinations of the kidney and liver of rats after 180 days of drug administration. (A) Represent liver and (B) Kidneys. The arrows make a representation of portal infiltration in the liver tissue and BVC (blood vessels congested) of the kidney tissue in treatment groups. The sections were stained with hematoxylin and eosin. *A. indica* AgNPs treatment group (30 mg/kg), H & E x400.

continuous use at 30 mg/kg may lead to damage of the liver, evident in both sub-acute and chronic studies. Portal hepatitis may suggest a chronic hepatitis of which the portal inflammation predominates. Recently, studies described mixed infiltrate of lymphocytes, neutrophils, monocytes, and occasional eosinophils, however the relative numbers of diverse cells and their relationship to disease progression are not known. The portal inflammation degree does not correlate with grade of lobular inflammation, but is associated instead with portal based changes, such as a ductular reaction (DR) (Gadd et al., 2014). The DR, a reactive lesion at the portal tract interface comprising small biliary ductules with a complementary complex of stroma and inflammatory cells which develops when hepatocyte regeneration is reduced and hepatic progenitor cell (HPC) proliferation takes charge (Gadd et al., 2014). HPCs are bipotential and capable of proliferation and differentiation into hepatocytes, to replace damaged cells. HPC activation and a DR are common responses to chronic liver injury

and are thought to precede progressive, portal fibrosis in which portal tracts, inflammatory cells and their mediators influence the differentiation and fate choice of HPC, which, in turn, may determine the balance between liver repair and fibrogenesis.

Both sub-acute and chronic toxicity studies indicated normal serum creatine and urea values, a share painter of non-nephrotoxicity. This is in agreement with the study by Shirodkar (2015). The kidney gross pathology and histological organization appeared normal generally. The renal architecture was maintained normal with glomeruli and tubules having normal structure at the dose level of 10 mg/kg. However, at the highest dosage (30 mg/kg), the organs showed an infiltration in cortex including glomeruli. Since the same observation was made for the control group rats, it may be lucid to speculate that the infiltration may not be associated with the drug administered.

The PCV values of rats observed in this study were within the normal range and they were comparable to the 28 days sub-acute values (37.5-47.7%), these corresponds with the recent results reported by Shamaki (2014) for normal albino rats. The observation that, there were no significant differences between the PCV of control nontreated and treatment groups indicates that administration of silver nanoformulated *A. indica* bark extract does not destroy blood cells after both 28 and 180 days of drug administration. These findings are comparable to the findings of Wen (2017) who reported that administration of silver nanoparticles in rats has no effect to the red blood cells after 28 days of drug administration.

Conclusion

The studies in toxicities of *A. indica* nanoformulation *in vivo* it currently gained extensive attention, and more knowledge on AgNP is required for safety evaluation and risk management. Taken our data and previous studies composed, it is rational to speculate that a lower dose of AgNP with extended exposure period could essentially accumulated in targeted organs and produce chronic toxicity. Additionally, the potential of carcinogenicity may intensify. Major concerns on *A. indica* AgNP's safety assessment at present are its disposition in the targeted organs and the subsequent toxicities, which necessitates a further genotoxicity and carcinogenicity study to follow. The distribution of *A. indica* AgNPs in animals can be identified by, for illustration, a recently developed method using gold nanocluster as fluorescence probes Zhang, 2015. In conclusion, this study sheds light on the underlying safety of *A. indica* AgNPs, targeted organs and genotoxicities particularly generated by *A. indica* AgNP in Swiss albino rat models. The specific immuno-toxicities and potential carcinogenic effect induced by nanoformulation need to be further investigated in a chronic toxicity study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

AgNPs, Silver nanoparticles; **ALT,** Alanine aminotransferases; **AST,** Aspartate aminotransferases; **BWT,** Body weight; **FTIR,** Fourier Transform Infrared Resorption; **KeV,** Kilo electron volts; **kg,** Kilogram; **NM,** Nanometer; **NPs,** Nanoparticles; **PCV,** Packed Cell Volume; **SEM,** Scanning Electron Microscopy; **SPR,** Surface plasmon resonance; **TEM,** Transmission electron microscopy; **UV/Vis,** Ultraviolet visible spectrum; **XRD,** X-ray diffraction analysis.

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Optimization of plant growth regulators for meristem initiation and subsequent multiplication of five virus tested elite sweet potato varieties from Ethiopia

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Vegetatively propagated crops are exposed to pests every growing season, providing them with the opportunity to feed on the plants and transmit diseases. Meristem culture is an important method to clean for viruses to restore high yields. Media optimizations with different concentrations and combinations of 6-benzylamino purine (BAP) and Naphthalene Acetic Acid (NAA) were tested for their effects on shoot initiation from meristems. This was followed by regeneration and shoot multiplication from nodal cuttings in five elite varieties of sweet potato. There was a highly significant genotype (variety) x environment (media) interaction for all experiments. Concentrations and combinations of BAP and NAA significantly affected the percentage of meristem survival/regeneration and subsequent shoot multiplication. The best combination of the plant growth regulators was NAA (0.1 mg/L) and BAP (1.0 mg/L). For further propagation, shoot numbers per single nodal cuttings were significantly affected by BAP concentrations ($p < 0.05$) and genotypes. The medium which gave maximum numbers of shoots was ½ MS media supplemented with BAP (2.5 mg/L) and GA_3 (0.5 mg/L). The tallest shoots, with the highest number of internodes, suitable for transfer to soil, was obtained from ½ MS media without any growth regulators for all of the cultivars.

Key words: Media, meristem, multiplication, regeneration, shoot, sweet potato.

INTRODUCTION

Sweet potato (*Ipomoea batatas* L. Lam) ranks as the fifth most important food for most developing countries (FAO, 2017). Africa stands second in total production of sweet potato and this production is increasing at a fastest global rate (FAO, 2017). Ethiopia is the sixth largest sweet potato producer in Africa. The crop is critical at both household and national levels for food security (FAO, 2017). However, the production of sweet potato suffers from virus infections which results in low national average yields (8.2 tonnes), far below the world average of 12.3 tonnes (FAO, 2017).

Tissue culture techniques have been used as a biotechnological tool for virus elimination and mass multiplication of vegetatively propagated crops, including

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sweet potatoes (International Atomic Energy Agency, 2004). Uniform and disease-free planting materials can be multiplied using tissue culture. Tissue culture may improve agriculture by developing quality-planting materials to meet the ever-increasing global demand for food and feeds. In addition, plant tissue culture techniques have a great potential for mass multiplication, conserving elite cultivars and rare cultivars treated to extinction using artificial media (Torres, 1989). Tissue-cultured plants often out-perform those propagated conventionally in productivity and also in the quality of produce.

Initiation and regeneration of an explant cultured on artificial nutrient media is influenced by the type and concentration of nutrients, plant growth regulators (PGRs) and their appropriate ratios, genotype and type of explants (Varshney and Anis, 2014) in addition to mother plant treatments prior to propagation *in vitro*. For the initiation of growth and development of an explant in any culture, in addition to the basic MS medium (Murashige and Skoog, 1962), the explant requires additional inputs and is highly affected by concerted and cooperative activities of PGRs, particularly auxins and cytokinins (Gaspar et al., 1996). The most important and most commonly used synthetic auxins in tissue culture are 2, 4-dichlorophenoxyacetic acid (2, 4-D) and naphthalene acetic acid (NAA) whereas that of cytokinins are kinetin and BAP (Gaspar et al., 1996). The concentrations and the combination between PGRs determine the morphogenetic responses towards shoot initiation.

Varieties of the same species often respond differently to artificial media (Abe and Futsuhara, 1986; Jan et al., 2001), through genotype x environment interactions. The success of sweet potato shoot initiation and multiplication in tissue culture may vary depending on the genotype used and the type and concentrations of PGRs supplemented (Dugassa and Feyissa, 2011; Wondimu et al., 2012; Abubakar et al., 2018). These previous studies show that media optimized for sweet potato cultivar are genotype-specific and if not optimized, may result in poor, or no, regeneration. In Ethiopia, several sweet potato varieties have been evaluated for traits of interest (yield, disease resistance, nutritional content, adaptability to locations) and released for use (Tofu et al., 2007; Gurmu et al., 2017). However, efficient and universal protocols for rapid multiplication of virus-free planting materials have not been optimized, except for a few of these cultivars (Dugassa and Feyissa, 2011; Wondimu et al., 2012). For this study, we have used five high yielding varieties of sweet potato released for different regions (southern, eastern and western) of Ethiopia. Three of them are white and the remaining two are orange-flesh sweet potato varieties (containing the valuable β-carotene, the precursor of vitamin A).

Our hypothesis is that these elite genotypes will vary in their responses to the PGRs. It is important to optimize the best media for each genotype. Moreover, we intend to clean the elite varieties from the infecting virus(es) and undertake further mass propagation of these clean genotypes for distribution to the farmers. The present study was carried out to (1) optimize the best combinations of PGRs to obtain a high rate of shoot initiation from meristem explants of four local varieties selected for their strong agronomic performances and (2) to optimize the appropriate concentrations of BAP for the shoot multiplication of five Ethiopian sweet potato varieties using single nodal cuttings. These two aims will, potentially, secure enough clean plant material for Ethiopian famers of these genotypes, since Ethiopia already has commercial tissue culture facilities in place for propagation of sugar cane with the capacity to take on disease-free sweet potato propagation as well. This piece of work contributes to the undergoing effort of cleaning the virus from elite variety and multiplying the virus-free sweet potato plants.

MATERIALS AND METHODS

Plant materials and experimental sites

Five high yielding Ethiopian sweet potato varieties, three of which ('Hwassa-83', 'Guntute' and 'Kulfo') were obtained from the Hawassa Agricultural Research Center, and two from Bako Agricultural Research Center ('Tola') and Haramaya University ('Berkume') were used for this study. The vines of the varieties were imported into Norway with a permit from the Norwegian Food Authority (Mattilsynet) for virus indexing and virus-cleaning. Plants were grown in quarantine rooms and used as stock to provide shoot tips for excising meristems. The studies on PGRs in the two media were conducted in the Plant Cell Laboratory at the Norwegian University of Life Sciences (NMBU), Norway. Meristem tip culture and single nodal cuttings were used as explant for shoot initiation and shoot multiplication experiments, respectively. The nodal cuttings used as explants for the multiplication experiments were from plants in tissue cultures grown on MS media without PGRs, as the last medium before the transfer to soil.

The virus-cleaned and virus-tested cultures were exported back to Ethiopia as tissue cultures where the acclimations were performed at Hawassa University.

Experimental design and treatment set-up

Medium preparation

The basal medium used throughout was Murashige and Skoog (MS) (1962) from Duchefa Biochemie (M0222.0001) containing 4.4 g/L (macronutrients, micronutrients, and vitamins). The MS medium was used in either full (initiation) or half concentration (multiplication). The media were all supplemented with 30 g/L sucrose and 6 g/L agar (Sigma-Aldrich, Spain). Plant growth regulators (PGRs) were supplied according to the two experimental set-ups described below.

Initiation of meristems

The experiments were set up as complete factorial experiments (4x4x4) with 16 media combinations of two PGRs (NAA and BAP)

Table 1. Plant growth regulator combinations for A: Initiation of meristems using NAA and BAP in 16 combinations (media 1-16) for all four genotypes: 'Guntute', 'Kulfo', 'Berkume' and 'Tola'. B: Regeneration of nodal cuttings of 5 combinations (media 17-21) and 5 genotypes: 'Guntute', 'Kulfo', 'Berkume', 'Tola' and 'Hawassa 83'.

A: 1/1 MS	**BAP (mg/L)**				
NAA (mg/L)	**0**	**0.1**	**1.0**	**2.5**	
0	1	2	3	4	
0.01	5	6	7	8	
0.1	9	10	11	12	
0.5	13	14	15	16	
B: ½ MS			**BAP mg/L**		
GA_3 (mg/L)	**0**	**0.5**	**1.5**	**2.5**	**3.5**
0	17		Not tested (NT)		
0.5	NT	18	19	20	21

(Table 1A, media 1-16) for each of the four varieties ('Berkume', 'Guntute', 'Kulfo' and 'Tola').

Regeneration of nodal cuttings

MS medium (1/2 strength) supplemented with five concentrations of BAP (0, 0.5, 1.5, 2.5, and 3.5) mg/L was combined with 0.5 mg/L of the natural gibberellin, GA_3 (Table 1B, media 17-21) and were tested to assess the response differences of the five sweet potato varieties ('Berkume', 'Guntute Kulfo', 'Tola' and 'Hawassa-83'). No PGR was included as a control. Shoot multiplication from each single nodal *in vitro* cutting was recorded for each combination.

Preparation of explants, surface sterilization and the establishment of a culture

Preparation of meristems

Shoots of about 2 - 3 cm were trimmed for leaves and parts of leaf petioles, the surface was then sterilized in 70% ethanol for about 30 s, and thereafter 10 min in a solution of 5% (v/v) sodium hypochlorite (VWR) and 0.02% v/v of Tween 20 (Sigma-Aldrich, St. Louis, USA). Finally, the shoot tips were rinsed three times with double distilled, autoclaved (sterile) water. The apical and the two adjacent axillary meristems from the top were excised under a dissecting microscope and placed on the various media under sterile conditions. Seven meristems were cultured on each of the 16 different combinations of BAP and NAA levels. Treatments were arranged in a completely randomized design (CRD) and each experiment was replicated three times. In total, 336 meristems per variety were cultured on the different combinations of BAP and NAA.

Regeneration of nodal cuttings for multiplication

In vitro cultures of five sweet potato varieties, which were first kept on hormone-free ½ conc MS medium for 6 weeks, were used as the starting material for this experiment. They were grown under the same growth room conditions as described below and transferred to ½ MS medium, with PGR combinations 17-21 (Table 1B) to evaluate shoot multiplication using single nodal cuttings. Four single nodal cuttings were cultured in each jam jar as a single experimental unit and each combination was replicated three times, that is, 12 single nodal cuttings of each of the varieties represented each treatment.

Culture conditions

All the *in vitro* cultured explants were grown under the same growth room conditions: temperature of 25 ± 1°C, a light intensity of 28 µmol m^{-2} s^{-1} and 16/8 h (light/dark) photoperiod provided by white fluorescent lamps (Osram L 58W/840 Lumilux), and a relative humidity of 70%.

Acclimatization

Cleaned, virus tested tissue culture plantlets were returned to Ethiopia from Norway with an issued Health Certificate according to the Convention of Biological Diversity (1993). The cultures were transferred to an insect-proof screen house at Hawassa University, Ethiopia into polyethylene plastic tubes filled with solarized top soil: compost: sand in a ratio of 3:2:1. Plants were covered with a plastic sheet for the first two weeks to maintain plant turgidity. One month after planting, the surviving plants were counted and a percentage of survival was calculated.

Data collections and subculturing

Shoot initiation

Cultures with infection symptoms (if any) were, immediately recorded and discarded. Meristems with a green color after two weeks of culture were counted as survived; whereas those that failed to turn green were considered to be dead. The percentage of meristem survival was determined by dividing the green meristems that survived by total cultured. After 6 weeks of incubation, the survived meristems were transferred into test tubes, which contained the same fresh media combination, and to allow for shoot regeneration. Twelve weeks later, the number of meristems that induced a shoot size of more than 1 cm, the number of meristems that produced callus, and the callus weight (mg) induced for each meristem combination were recorded.

For regeneration of nodal cuttings experiment, culture survival, the number of shoots induced per nodal explant, the length of shoots, the callus weight per nodal explant, and the number of nodes were counted after four weeks.

Table 2. Differences in responses of Ethiopian sweet varieties to basic MS media, supplemented with NAA and BAP, calculated after 12 weeks of culture and as an average over all media combinations.

Variety	Mean survival of meristem (%)*	Mean shoot initiation from meristems (%)*	Mean callus weight produced per meristem* (mg)
'Kulfo'	84.3^{ab}	39.1^{a}	261.0^{a}
'Berkume'	83.3^{a}	24.1^{b}	248.6^{a}
'Tola'	74.7^{ab}	15.2^{b}	236.7^{a}
'Guntute'	71.1^{b}	16.1^{b}	147.0^{b}

*Mean values in the same column followed by same superscripts letter are not significantly different at $P < 0.05$ according to Tukey pairwise comparisons.

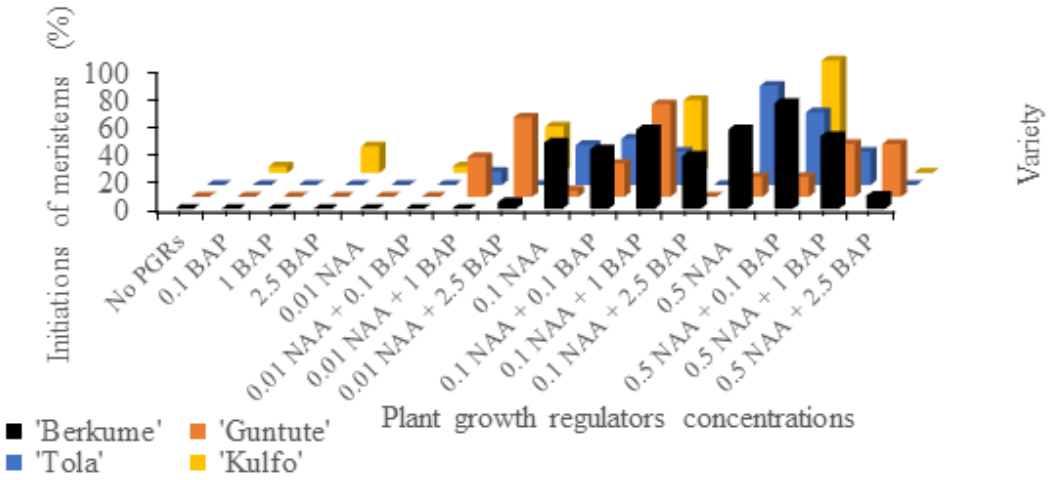

Figure 1. The average percentage of initiations shoot from meristem combined for four sweet potato varieties from Ethiopia as affected by 16 different concentrations and combinations of plant growth regulators (NAA and BAP) supplemented to MS medium.

Data analysis

All of the data were subjected to ANOVA (General Linear Model) using Minitab Statistical Software version 18 (Minitab Inc., Pennsylvania, U.S.A). Means that were significantly different were separated using a Tukey LSD test at the probability level of 5% ($p \leq 0.05$).

RESULTS AND DISCUSSION

Genotype and PGR effects on meristem survival and further shoot development

Different concentrations and combinations of NAA and BAP had no significant effects on the survival of the meristems after two weeks; they greened in a similar way for all PGR combinations (data not shown). As time went by (a further 16 weeks), the different concentration and combinations of PGRs gave significantly different survival responses amongst the four varieties tested (Table 2). Many meristems did not develop further into shoots and eventually died, particularly when no NAA was added (Figure 1). We can safely conclude that auxin is needed for meristem survival of sweet potato. This is in accordance with previous findings by Dugassa and Feyissa (2011).

'Kulfo' obtained the highest shoot initiation rate (89%) after a further 16 weeks on the MS media supplemented with 0.01 mg/L NAA and 1.0 mg/L BAP (medium 7) (Figure 1). This is the same optimal medium as 'Berkume', while 'Tola' preferred a medium with only NAA (0.5 mg/L) and 'Guntute' performed best on 0.1 mg/L NAA + 1 mg/L BAP. On average across all of the media, the highest survival rate of 83% was recorded for 'Kulfo', while the lowest (of the media with growth) was for 'Guntute' (71%) (Table 2). It is reported that two or more hormones (BAP and NAA in this case) can interact synergistically or antagonistically in many circumstances (George et al., 2008).

When comparing our study with that of Dugassa and Feyissa (2011), we have included a wider variety of genotypes from several regions of Ethiopia. Our aim was to include the best varieties from different sweet potato producing regions in Ethiopia to be able to clean elite

Table 3. Analysis of variances for the effect of media, variety (genotype) and media x variety interaction on regeneration of sweet potato shoots *in vitro*.

Source	Var	SE Var	Z-value	P-value
Media	259.70	128.82	2.02	0.022
Variety	71.11	85.36	0.83	0.022
Media*Variety	223.69	69.87	3.22	0.001
Error	207.73	27.76	7.48	0.000
Total	762.22			

Ethiopian varieties from viruses. Our results are in agreement with the results of Dugassa and Feyissa (2011) for the two varieties that they tested. We have demonstrated the optimal PGR combinations for improved initiation of shoots from shoot tip meristems of three more varieties. In descending order of response: 'Kulfo', 'Berkume', Guntute' (same as Dugassa and Feyissa, 2011) and 'Tola' as the least responsive on these media. Dugassa and Feyissa (2011) noted that 'Guntute' had the highest response rate of the three varieties tested, on their media combinations. However, we have tested a much higher level of NAA, combined with the same levels of BAP, and found that their optimal NAA (0.01 mg/L) had not reached an optimum for any of the varieties that we tested, and 'Guntute' was tested in both studies. Our overall optimal NAA level was x10 higher (0.1 mg/L), combined with 1.0 mg/L BAP. The BAP optimal for all varieties combined (Figure 1), on the other hand, is in accordance with Dugassa and Feyissa's findings (2011). We can conclude that the optimal medium for a new variety would be to try out this combination (NAA 0.1 mg/L + BAP 1.0 mg/L).

The picture is different when looking at each variety separately, since we have a clear genotype x environment interaction (Figure 1). This shows that each variety has their own requirements, as previous reports have also concluded for both Ethiopian (Dugassa and Feyissa, 2011) and West African varieties (Addae-Frimpomaah et al., 2014). This kind of factorial experiment, to reveal the optimal concentrations of PGRs on meristem initiation and regeneration has been confirmed by Su et al. (2011). The number of weeks to induce shoots varied for the varieties; 'Kulfo', 'Hawassa 83' and 'Berkume' took 5 to 7 weeks, while 'Tola' and 'Guntute' took 3 weeks longer to respond.

The statistical analysis shows that the interaction in the response of meristems, between the varieties and PGR combinations, was highly significant (p=0.001), while the response was only significant, at a level of 5%, when testing the varieties and media independently (Table 3).

Figure 1 illustrates the best concentration and combinations of BAP and NAA for shoot initiation media. The details of the statistics behind this figure are presented in Online Resource 1. No, or low, concentration of PGRs did not result in shoot regeneration, as with the initiation meristems. Both 'Kulfo' (81%) and 'Berkume' (76%) had the highest percentage of meristems giving shoots on the same PGR combination: MS with 0.5 mg/L NAA and 0.1 mg/L BAP (medium 14). 'Guntute' performed best on MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA (medium 7) with (67%) of the meristems producing shoots. This is the same result that Dugassa and Feyissa (2011) obtained for 'Guntute', except that they also added 1 mg/L GA_3 in their medium, in addition to BAP and NAA. We can conclude that adding gibberellin was not necessary in our case. The maximum regeneration from meristems in 'Tola' was 72%, on yet another medium: 0.5 mg/L NAA without any added BAP (medium 13). These three genotypes were not part of the study by Dugassa and Feyissa (2011), so this is the first report of their optimal combinations. Preliminary studies (data not shown) with 'Hawassa-83' was also consistent with Dugassa and Feyissa (2011), so we decided to omit this genotype from the large factorial media experiment, but kept 'Guntute' for reference. Other studies, using other genotypes, have also reported on the effect of variety, the concentrations and combinations of PGRs on the regeneration capacity of sweet potato plants from meristems (González et al., 2008; Sivparsad and Gubba, 2012; Alam et al., 2013; Mbinda et al., 2016). So, it is not surprising that the regeneration of sweet potato plants from meristem cells varies depending on the tissue culture media components (PGRs, nutrients, vitamins, sugar, agar), genotypes and the culture conditions (Dugassa and Feyissa, 2011; Addae-Frimpomaah et al., 2014). Already in 1957, Miller and Skoog revealed the importance of auxin and cytokinin concentrations and their combinations for cell proliferation and new organ regeneration in tobacco. Since then, this kind of factorial experiment, to reveal the optimal concentrations of PGRs, has been repeated by numerous authors for other plants. When optimizing for a species with genotype x media interactions, we believe it is important to perform such an exercise. It is necessary to be sure that we have the best possibility of obtaining surviving meristems when the purpose is to clean a particular genotype for viruses, to increase the chance of obtaining a clean plant. From our results for the initiation of shoots from meristems for all varieties, it averaged between a low of 1.2% on 1 mg/L BAP to a high of

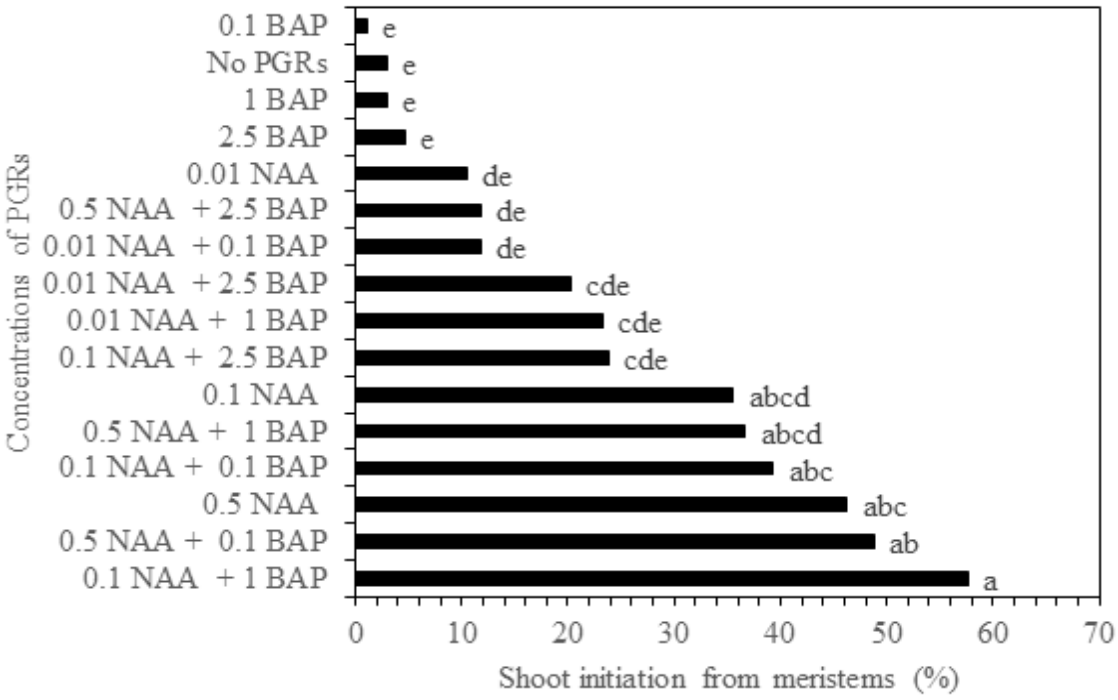

Figure 2. The average initiations of shoot tip meristems of four Ethiopian sweet potato varieties on basic MS media supplemented with 16 different concentrations of NAA and BAP. Mean (represented by the bars) that do not share the same letter are significantly different at 5% level.

57.7% on 1 mg/L BAP and 0.1 NAA (Figure 2). Moreover, this media combination induced an average low callus weight of 98 mg/meristem, which is desired to reduce the risk of somaclonal variation. Therefore, this combination of 1 mg/L BAP and 0.1 NAA could be the one to use for new cultivars, if a factorial PGR experiment is not feasible.

PGRs combinations and the varieties, when analyzed separately, both had statistically significant effects ($p<0.05$) on the initiations of shoot from meristems (Table 2). When considering overall performances for all tested genotypes, 0.1 mg/L NAA and 1 mg/L BAP (medium 11) was the best concentration and combination tested that regenerated shoots in 58% of the cultures (Figure 2). Figure 2 reveals that media 9, 15, 13, 14 and 11 are not significantly different at a level of 5% but do show an increasing level of regeneration when averaging over all genotypes. Significant differences, in respect of days for shoot induction, were also observed. 'Kulfo' and 'Berkume' took 5 to 7 weeks to regenerate shoots of about 1 cm or more, whereas 'Tola' and 'Guntute' took between 8 to 10 weeks (data not shown).

PGRs and genotypes highly affected shoot multiplication and height of nodal explants

The factorial media experiment using nodal explants further emphasized the importance of such media experiments. Table 4 shows the highly significant effects of the five media combinations, the genotypes on shoot multiplication from nodal cuttings, as well as the interaction between them.

The sweet potato nodal cuttings responded well to transfer from *in vitro* cultures when propagated on to hormone-free ½ MS medium. As expected, the survival rate was between 95 to 100% in all cases.

Figure 4A shows that the highest mean number of shoots for all varieties were obtained when supplying either 1.5 or 2.5 mg/L of BAP combined with 0.5 mg/L GA_3 (medium 19 or 20). Figure 3 provides more details for the optimal medium for each cultivar. 'Berkume', 'Hawassa' and 'Guntute' produced the maximum mean number of shoots of 2.8, 1.7 and 1.8 correspondingly on ½ basic MS (medium 20) supplemented with 2.5 mg/L of BAP and 0.5 mg/L GA_3 (Figure 3). However, 'Kulfo' produced the highest mean shoot number of 1.6 at a concentration of 3.5 mg/L BAP and 0.5 mg/L GA_3 (medium 21). 'Berkume' produced a significantly higher number of shoots, compared to the other four varieties, on average over all media tested (Figure 4B). 'Tola', essentially, did not multiply on any of these media, as it produced only, or at the very best, 1.1 shoot per nodal cutting (medium 21) in one month (Figure 3).

In this study, increasing the concentration of BAP significantly decreased the height of the shoots induced

Table 4. Analysis of variance for the number of sweet potato shoots obtained from singles nodal cuttings *in vitro.*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Media	4	5.578	1.3946	9.56	0.000
Variety	4	8.795	2.1987	15.08	0.000
Media*Variety	16	8.522	0.5326	3.65	0.000
Error	50	7.292	0.1458		
Total	74	30.187			

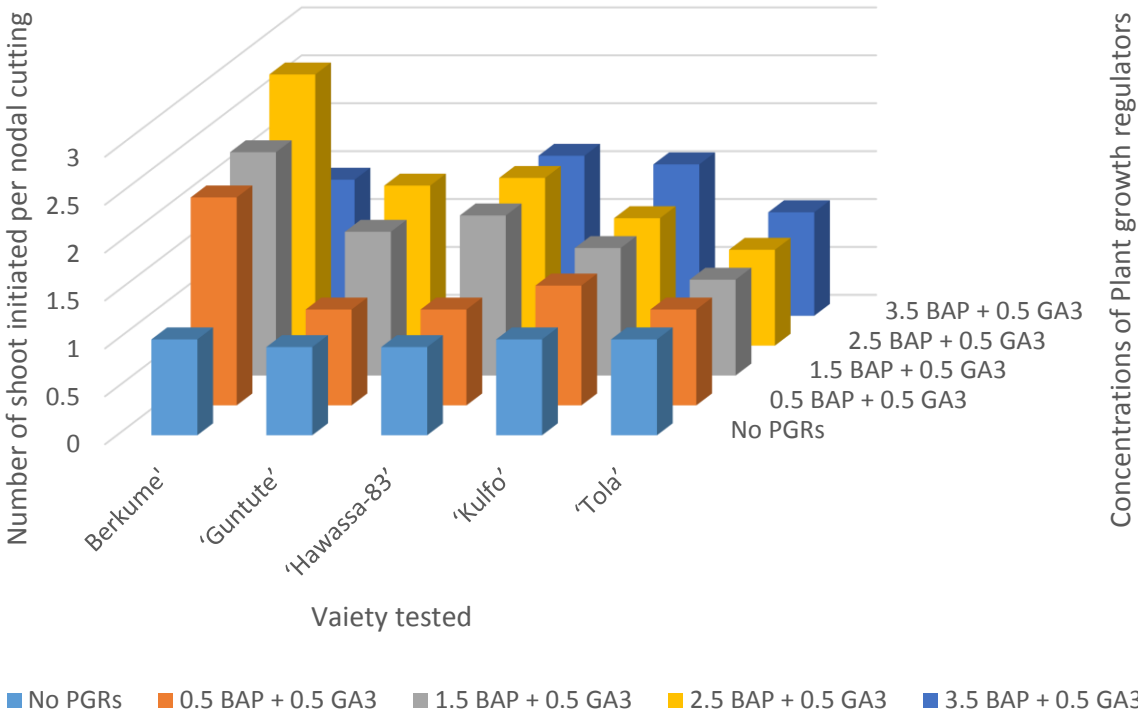

Figure 3. The effect of media combinations and varieties on number of shoots initiated per nodal cutting of five Ethiopian cultivars from different regions.

from the nodal cuttings in all of the studied varieties (Figures 4A and 5). The results agreed with the findings of the previous studies conducted that evaluated the effect of BAP concentration on nodal cuttings regenerated using sweet potato from Ghana and Ethiopia (George et al., 2008; Dugassa and Feyissa, 2011; Addae-Frimpomaah et al., 2014). Interestingly, the lowest mean number of shoots and the shortest mean shoot height was obtained from 'Tola' among the other varieties that were tested in response of the BAP concentrations. This could agree with the suggestion that the effect of hormones on any developmental process depends on the species (George et al., 2008). However, the highest (6.9 cm) and shortest (1.68 cm) mean shoot height were registered for 'Tola' and 'Berkume' cultured in basic ½ MS media, without PGRs (Table 4 and Figure 5). Furthermore, this study also showed that varieties responded differently to the same concentration of BAP and resulted in varying mean shoot number. This can be explained by the suggested theories that the effects of plant growth regulators vary depending on culture conditions, explant types and the genotype (Gaspar et al., 1996).

Nodal cuttings planted on MS media without BAP generated the highest shoots compared to any other levels of BAP concentration we tested. It appears that nodal cuttings cultured on different BAP levels induced shoots of varying number and length (Dugassa and Feyissa, 2011). The higher shoot length can provide more cuttings per plant and can compensate for the number of shoot induced by adding BAP. We suggest that using ½ MS media without PGRs could be good for shoot multiplication of sweet potato varieties as it avoids costs of PGRs and gives longer shoots that can provide more nodal explant for multiplication.

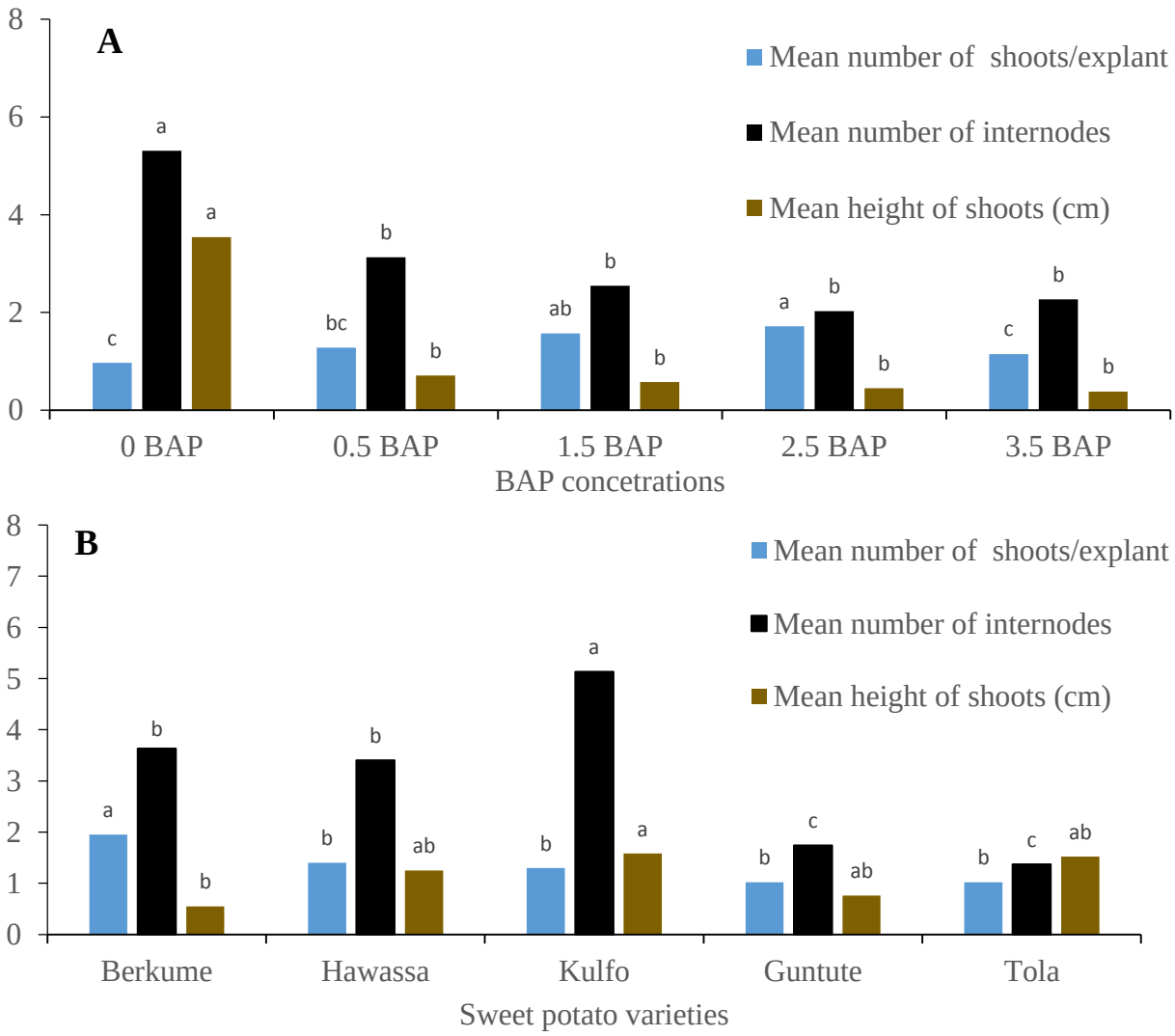

Figure 4. Mean shoot multiplication parameters (shoot number, internode number and shoot height) as affected by varying concentrations of BAP (each combined with GA_3 of 0.5 mg/L) for five elite sweet potato varieties from Ethiopia. **A**: BAP concentrations affected the parameters of shoot multiplications; **B**: varieties affected the shoot multiplication parameters. Bars represent mean values. The same colored bars followed by same superscripts letter are not significantly different at $P < 0.05$ according to Turkey pairwise comparisons.

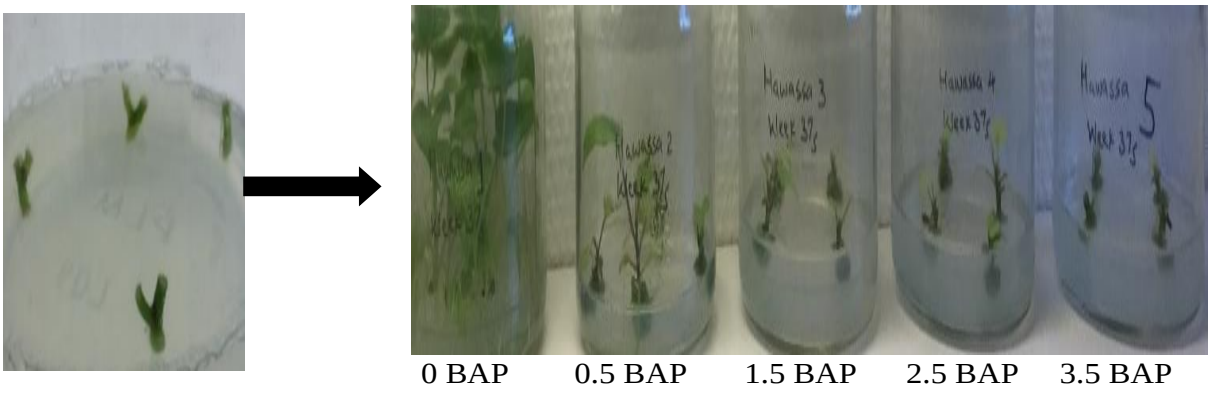

Figure 5. The shoot height of sweet potato *in vitro*, from the start (left), decreases with increasing concentrations of BAP.

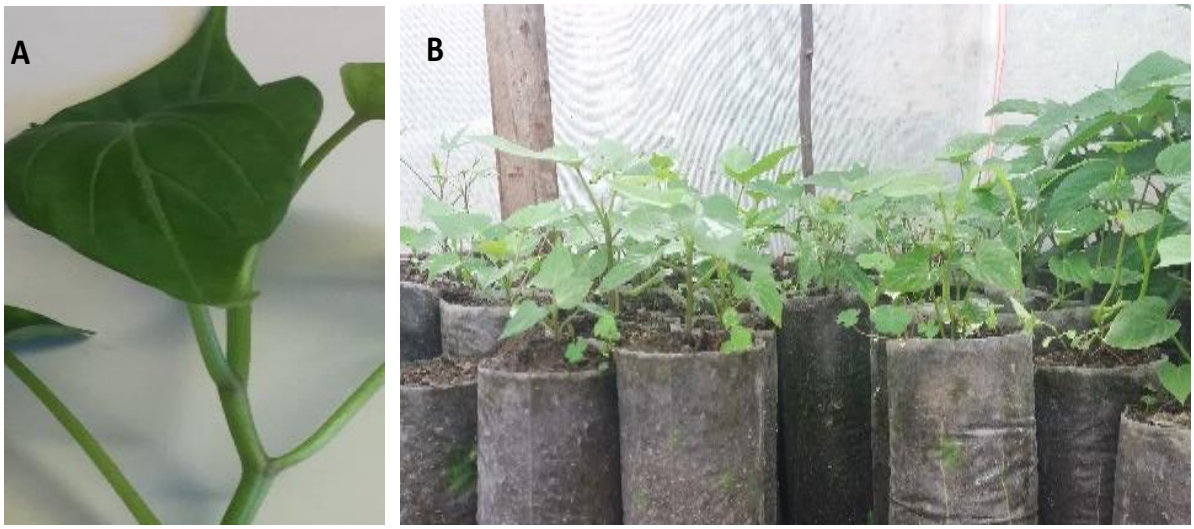

Figure 6. Establishment of tissue culture plants in soil. **A**: Shoot of 4 - 5 internodes from tissue culture used for planting. **B:** Examples of plants that survived were acclimatized and became established.

The tallest shoots and highest number of internodes were obtained on ½ basic MS media without BAP or GA_3 (hormone-free medium 17). Shoot length declined with increasing concentration of BAP for most varieties (Figures 4A and 6). 'Kulfo' obtained the tallest shoots of all varieties (Figure 4B) on average over all media tested. 'Tola', however, had the tallest shoots of all, 6.90 cm on the hormone-free medium 17 (Online Resource 4). Taller shoots secure a better transfer to soil and are, therefore, beneficial.

PGR and genotype affect internode number

The BAP concentrations (Figure 4A) as well as varieties (genotypes) tested (Figure 4B) significantly affected the number of internodes per single initial nodal explant. The number of internodes gives an indication of the potential of mass propagation since there will normally be at least one axillary bud for each node. Basic ½ MS media without BAP nor GA_3 (medium 17) gave the highest average (over all genotypes) internode number of 5.3, whereas the minimum (2.0) was obtained with a concentration of 2.5 mg/L BAP and 0.5 mg/L GA_3 (Figure 4A). The highest average internode number obtained from the various genotypes was 5.1 from 'Kulfo', while the lowest was 'Tola' with 1.4 internodes from each initial nodal cutting (Figure 4B).

No significant differences between varieties were recorded on the establishment rate (survival) when the tissue cultures of sweet potato plants were planted in screen house. In all of the varieties, more than 90% of the planted plants survived the acclimation and successfully established into mature plants within one month after planting (Figure 6).

PGR x genotype interactions for callus growth

This research did not set out to produce callus but it was recorded as a way of describing the responses to the media combinations. Also, callus has a potential use for the development of other regeneration methods, such as somatic embryogenesis protocols. In our case, this is an undesired feature, as we aim for shoots, not callus. Regeneration of shoots from callus is more prone to somaclonal variation than direct shoot organogenesis (Krishna et al., 2016), while the differentiation into somatic embryos probably requires an intact genetic constitution to facilitate the complex differentiation into somatic embryos. This hypothesis is supported by the work in poinsettia (Geier et al., 1992) who documented a higher variation in callus than in the subsequent somatic embryos.

The mean weight of callus induced per meristem was significantly affected by the concentrations and the combination of NAA and BAP in the varieties studied. The interaction of PGRs and varieties was significant at $p < 0.05$ for callus weight. Overall, on average across all varieties, the highest average callus weight (654 mg) was obtained from meristems cultured on basic MS medium (medium 16) supplemented with combinations of 0.5 mg/L NAA and 2.5 mg/L BAP (Figure 4). Medium 16 produced the highest callus weight for all varieties; 'Berkume' obtained a maximum mean weight (864 mg) of callus per culture, while 'Kulfo' obtained 643 mg, 'Tola' 642 mg, and 'Guntute' had only 465 mg (Figure 7, with statistics in Online Resource 2).

Meristems cultured on a basic MS media, supplemented with all concentrations of BAP but without NAA did not induce callus in any of the varieties (Figures 7 and 8, Online Resource 2). 'Berkume' was the culture with

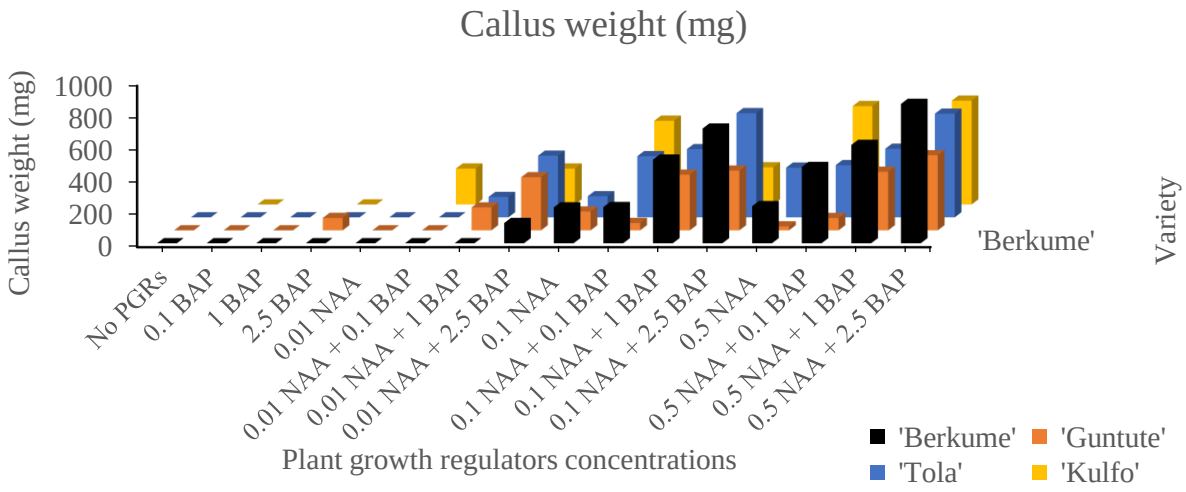

Figure 7. Effects of 16 combinations of NAA and BAP on the weight of callus induced from meristem explants of four sweet potato varieties from Ethiopia, after 12 weeks.

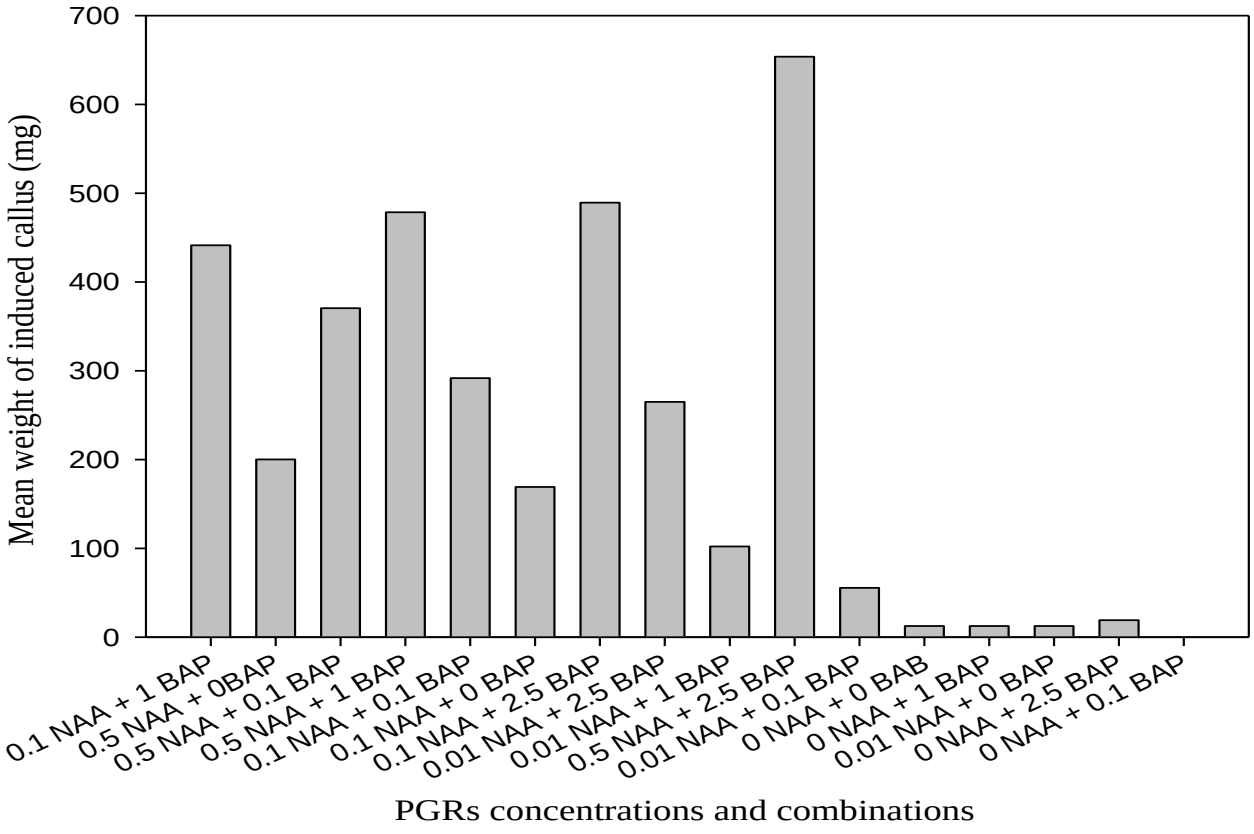

Figure 8. Mean weight of callus (mg) for four Ethiopian sweet potato varieties induced on 16 combinations of PGR (NAA and BAP) concentrations in MS media.

the highest callus mass that quickly outgrew the shoot bud.

The differences in weight of the callus measured between the varieties that we tested on the specific media concentrations and combinations could be explained by the differences in the amount of endogenous auxins in each variety (Onwubiko et al., 2015). Therefore, in order to improve the growth of the cultures, these authors

suggested PGRs that supplemented the basic MS media and should be in line with the concentration of endogenously PGRs in tissue. While this may be the explanation for the genotype variation, we would recommend testing a variety of PGR combinations systematically, rather than the lengthy process of determining the endogenous hormone content.

CONCLUSION AND RECOMMENDATIONS

This study demonstrated that different varieties of sweet potato respond very differently to the shoot initiation and shoot multiplication media, with respect to PGRs. For initiation of meristem, sweet potato have a clear requirement for auxin (NAA) without this, the addition of cytokinin (BAP) at any concentration has no effect. Then, the combinations of auxin and cytokinin are vital for meristem initiation and development into shoots. For the establishment of a novel genotype that did not form part of these experiments, the best chance of success would be to use MS medium supplemented with 0.1-0.5 mg/L NAA and 1.0 mg/L BAP.

For further multiplication *in vitro* of virus-free material, the best medium for 'Berkume', 'Guntute' and 'Hawassa-83', would be 2.5 mg/L BAP combined with 0.5 mg/L GA_3 and *no auxin*, would be the medium to try for new genotypes. However, 'Kulfo' preferred higher amounts of BAP (3.5 mg/L and 0.5 mg/L GA_3) to reach its highest potential. Since this is the highest amount of BAP used in this study, the optimum may be even higher. The same is probably true for 'Tola', which failed to multiply under our conditions, but still had the highest score on the same medium.

For all five of the tested varieties, the longest shoots with a higher number of internodes was obtained when single nodal cuttings were cultured on basic ½ MS media without any PGRs. These shoots are suitable for transfer to soil. We, therefore, recommend using hormone-free medium as the last medium prior to transfer to soil, to produce longer shoots with a greater chance of survival.

With this study, we have provided initiation medium, multiplication medium and elongation medium for elite sweet potato genotypes from Ethiopia, which can be used for multiplication of elite virus-free sweet potato varieties, and then distributed to the farmers or private multipliers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Selection for and biochemical characterization of DDT resistance in laboratory strains of *Anopheles arabiensis*

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Resistance to conventional insecticides still constitutes a major obstacle to control of malaria vectors. Xenobiotic pollutants encountered by aquatic stages of natural populations of malarial vector species in agricultural and domestic environment are often selected due to resistance to various insecticides. The Laboratory Matatuine (MAT) and Kayamba (KGB) strains of *Anopheles arabiensis* were subjected to controlled dosages of DDT for over twenty generations. WHO insecticide susceptibility protocols was used to monitor changes in mortality between generations. The selected lines of both strains developed resistance to DDT and cross resistance to permethrin. Polymerase Chain Reaction (PCR) detection of knock-down resistance (kdr) gene and sequencing revealed absence of L1014F mutations. Biochemical analysis of detoxification enzymes showed significant Glutathione S transferase (GST) activity in the selected lines [MAT: 0.236 (P>0.001) and KGB: 0.221 (P>0.014)], thus suggesting the presence of GST-based resistance mechanism.

Key words: Dichloro-diphenyl-trichloroethane (DDT), *Anopheles arabiensis.*

INTRODUCTION

Anopheles arabiensis is the second most efficient malaria vector species of the *An. gambiae* complex and it occurs in sympatry with *An. gambiae* sensu stricto in most areas. The two species form the most efficient malaria vectorial system in Africa (Powell et al., 1999; Coetzee et al., 2013). It often breeds in pesticide contaminated rice irrigation ecosystems found at malaria endemic areas in East and West Africa and adapt faster to man-made ecological habitats in urban cities (Coluzzi et al., 1979; Ijumba and Lindsay, 2001; Kamau and Vulule, 2006). It contributes considerably to malaria transmission in South Africa, Sudan, Nigeria and is the major malaria vector in Tanzania (Onyabe and Conn, 2001; Nardini et al., 2013; Matawo et al., 2014; Tarig et al., 2018). Current global strategy for control of malaria is based on chemoprophylaxis, treatment of diagnosed infected

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persons using effective anti-plasmodial drugs and insecticidal based methods to control the malaria vectors. The World Health Organisation (WHO) Pesticides Evaluation Scheme (WHOPES) approves eleven insecticides including permethrin and DDT to be used in public health, particularly malaria control programmes (WHO, 1998). Development of resistance to insecticides by malaria vector species still remains the major obstacle to malaria control globally (Karunaratne et al., 2018; Ranson et al., 2011). Resistance is the ability of an insect to withstand toxic effects of an insecticide by means of natural selection and mutations and is a heritable genetic trait passed down through generations (Davidson, 1957; Corvel and Nguessan, 2013). Increasing selection pressure on malaria vector populations caused by xenobiotic pollutants presence in agricultural and domestic environments necessitates selection for resistance to various insecticides (Nkya et al., 2012; Matawo et al., 2015). Resistance to DDT in field populations of *An. arabiensis* began to appear in South Africa (Hargreaves et al., 2003), Nigeria (Kristian et al., 2003), and subsequently, in other countries. Resistance to deltamethrin, permethrin and DDT have been reported in Ethiopia (Balkew et al*.,* 2010; Yewhalaw et al*.*, 2011) and Sudan (Abdullah et al., 2008).

The resistance to DDT in mosquitoes is generally associated with one of two mechanisms; increasing DDT dehydrochlorination catalysed by GSTs or decreased target site sensitivity (Hemingway and Ranson, 2000; Ranson et al., 2011; Karunaratne et al., 2018). Some of the previous studies on insecticide resistance in *An. arabiensis* have used populations selected in the laboratory for many generations (Hemingway, 1983; Matambo et al*.,* 2007). The use of laboratory strains (MAT and KGB) in comparison with field strains to study resistance mechanisms advantageously exclude factors such as effect of temperature, larval diet and exposure to agricultural pesticides that can confound diagnosis (Nardini et al*.,* 2013). The present study was aimed at selecting MAT and KGB laboratory reared strains of *An. arabiensis* for DDT resistance. The specific objectives were to (i) test for succeptibility of parental lines of MAT and KGB strains to DDT (ii) select subsequent generations for resistance to DDT (iii) test the selected lines for cross resistance to permethrin, and (iv) compare levels of activity of the detoxifying enzymes GSTs, esterases and monooxygenases in mosquitoes sampled from parental and selected lines of both strains.

MATERIALS AND METHODS

Establishment of *An. arabiensis colony*

Eggs of *An. arabiensis* MAT strains were collected from a field site at Matatuine located 10 km from Maputo in Mozambique. The colony was first established at the Institute Nacional de Saude Mozambique in May 2000 and transferred to The Liverpool School of Tropical Medicine in 2002. No information was available on the resistance status of this colony to any class of insecticide. Adult females of *An. arabiensis* KGB stains were caught at Kayamba, Zambesi Valley in Zimbabwe in 1975 and a colony established at The South African Institute for Medical Research. Eggs were brought on request to Liverpool in September 2004 and a colony was re-established there.

Mosquito rearing

The colonies of MAT and KGB *An. arabiensis* mosquito strains were maintained in the insectaries at the Liverpool School of Tropical Medicine. The mosquitoes were reared at temperature range of 27 to 28°C , 80 to 85% relative humidity with 12-h day/night light regime and 45-min dusk/dawn cycles. The duration of development from eggs to emerging adults ranged from 7-12 days amongst both strains. All mosquito larvae were fed on Tetramin fish food flakes, using ground-up flakes for the first instar. Extreme care was taken to avoid contamination between the different lines and strains of *An. arabiensis.* All larval trays were cleaned with hot water after each rearing cycle, when all the pupae had emerged into adults. Pipettes, egg pots and larval trays were colour-coded for each line of *An. arabiensis* strains. Adult mosquitoes were constantly provided with cotton wool soaked in saturated 10% sugar solution formed by using tap water. Females of both strains were given guinea pig blood twice a week (Hunt et al., 2005). Samples of the adult mosquitoes were identified to species using the polymerase chain reaction method described by Scott et al*.* (1993).

Susceptibility to WHO bioassays

Adult mosquitoes from both colonies of the parental lines of *An. arabiensis* MAT and KGB strains were tested for susceptibility to DDT. Bioassays were performed according to WHO protocols using standard WHO susceptibility test kits and 4% DDT impregnated papers (WHO, 1998). Survivors from each test were placed in a separate cage and used to establish subsequent generations.

Selection for resistance to DDT

Mosquitoes from *An. arabiensis* MAT and KGB strains, which survived previous exposures to DDT, were reared and their progeny subjected to selection using 4% DDT. Adult mosquitoes from *An. arabiensis* MAT strain were maintained under selection pressure with 4% DDT continuously for eight months and selection was interrupted for five months due to a crush in the colony, but thereafter the selection pressure was continued further for eight months. Mosquitoes from *An. arabiensis* KGB strain were similarly selected for resistance to DDT for a period of twenty three months without interruption.

Testing for cross resistance to permethrin

Batches of mosquitoes from *An. arabiensis* MAT and KGB selected lines were tested for resistance to DDT and cross-resistance to permethrin. Samples of adult mosquitoes from F10 and F20 selected generations of *An. arabiensis* MAT and *An. arabiensis* KGB respectively were exposed to 4% DDT and 0.75% permethrin for one hour. Knocked-down mosquitoes were recorded at intervals of ten minutes. The KDT50 and KDT90 knock-down times were calculated by probit analysis. The data was entered into Minitab 14 and LDP line software programmes for the analysis.

Knockdown resistance (kdr) assay

A PCR assay described by Martinez-Torres et al*.* (1998) was used to

Table 1. Susceptibility of adult mosquitoes in parental MAT colony to DDT.

Time (min) exposure	Number tested	Number dead	Number alive	% Mortality 24 post-exposure
15	58	16	42	27.5
30	80	53	27	66.3
45	135	116	19	85.9
60	65	57	8	87.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=338) sampled from the F1 generation of parental line.

Table 2. Susceptibility of adult mosquitoes in parental KGB colony to DDT.

Time (min) exposure	Number tested	Number dead	Number alive	% Mortality 24 post- exposure
15	67	13	54	18.6
30	85	38	47	44.7
45	98	65	33	66.3
60	148	121	27	81.6

Initial scores for mortality from WHO diagnostic test kit for 4% DDT tested against adult mosquitoes (n=398) sampled from the F1 generation of parental line.

test for the presence of the typical kdr mutations in individual mosquitoes sampled from parental and selected lines of both strains.

Biochemical assays

Unexposed mosquito samples taken from the parent stock and F15 selected generation of *An. arabiensis* MAT and KGB strains were kept at -80°C for biochemical analysis. Biochemical assays were performed according to the standardized procedures described in the manual by Hemingway (1998). Batches of 22 one-day old, frozen mosquitoes were individually homogenized in 200 µl of distilled water in 1.5 ml Eppendorf tubes. The crude homogenate was spun at maximum speed of. 10,000 rev min^{-} for two minutes in a microfuge. After centrifugation, the supernatant from each Eppendorf tube was then transferred to a well of a micro titre. Esterases, monooxygenases and GST assays were carried out in line with WHO (1998).

Protein assay

Protein assays were conducted according to the method of Bradford (1976). Microfuged homogenate (10 µl) from each mosquito was added to 300 µl Bio-Rad Protein assay reagent (diluted 5 times from stock), incubated for 5 min and end point absorbance measured at 570 nM. Protein concentration was determined by converting the absorbance into concentration based on a bovine serum albumin standard curve.

RESULTS

WHO susceptibility assays

The susceptibility levels to DDT of adult mosquitoes of the original parental populations of the *An. arabiensis* MAT and KGB strains were determined. A total of 338 adult mosquitoes aged two to three day old from the parental *An. arabiensis* MAT strains were exposed to 4% DDT for different time periods (Table 1). The 87% mortality after exposure to DDT for one hour indicates the presence of low level of resistant genotypes in the MAT parental colony.

In the KGB strain, 389 adult mosquitoes in batches of 20 to 25 were exposed at the four different time points and mortality was recorded 24 h post exposure as shown (Table 2).

The 81.6% mortality after exposure to DDT for 1 h suggests higher level of resistant genotypes in KGB than in the MAT colony.

Selection of resistant genotypes

The mortality decreased from 73.5% in the F3 to 51.4% in the F6. Due to rearing problems, selective pressure was not applied in generations from F7 to F13. The mortality rose back to 61.8% in F14 but decreased gradually to 48.3% in generation 16. Selection at 45 min exposure period raised the mortality to 69.4% but subsequently decreased to 53% in F20 (Figure 1).

The KGB colony did show a similar pattern of response to DDT. However, the selection pressure was gradually increased from 30 min in F1 generation to 60 min over 20 generations. Over these selected generations, the mortality decreased from 56.4% in F6 to 28.4% in F14 (Figure 2).

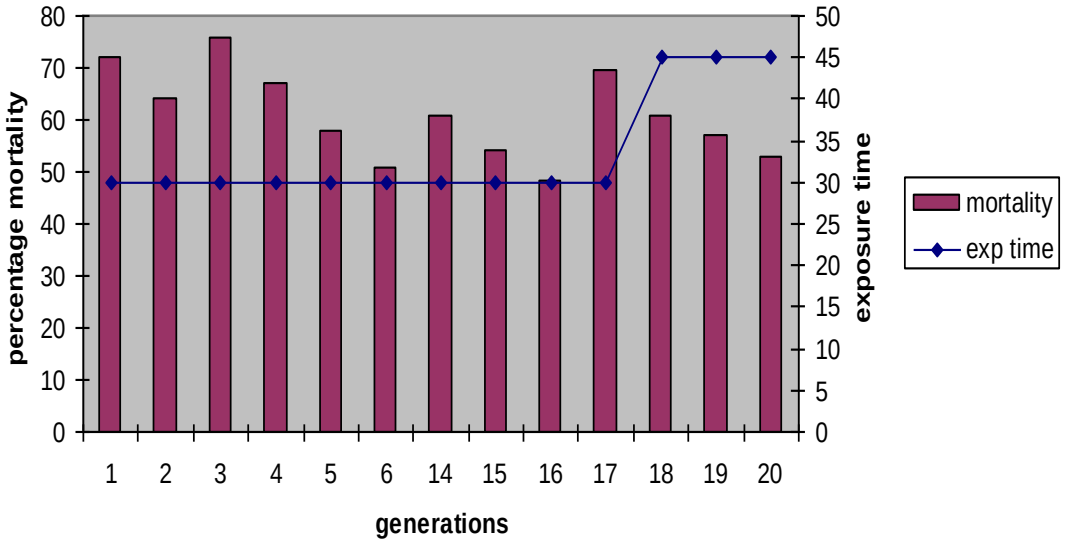

Figure 1. Selection of *Anopheles arabiensis* MAT strain with DDT.

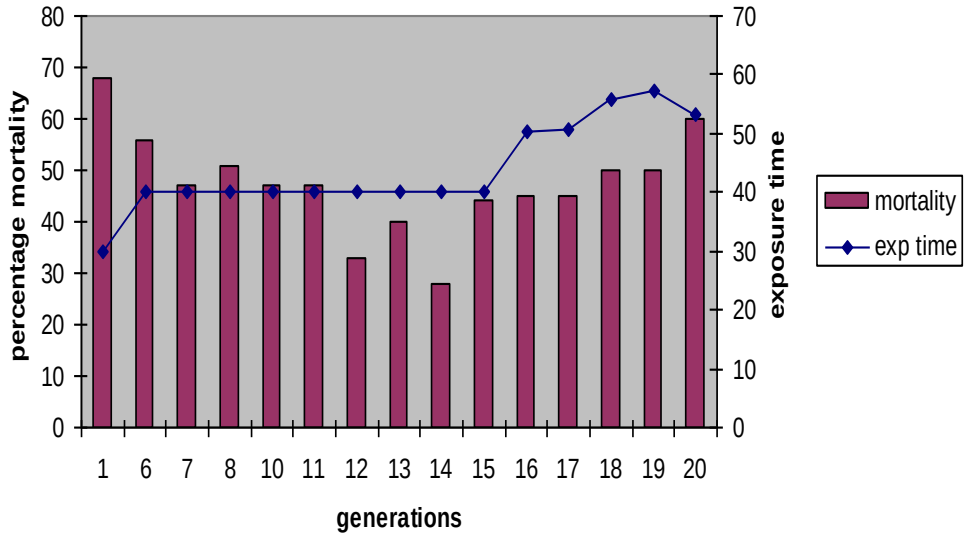

Figure 2. Selection of adult *Anopheles arabiensis* KGB strain with DDT.

After selection, the susceptibility tests with the diagnostic dose of DDT (4%) were repeated at different time points for the parental and selected populations of both *An. arabiensis* MAT and KGB strains. The LT50 values for DDT were 23.4 min and 33.2 min (resistant ratio 1.4) in the parental and selected colonies of *An. arabiensis* MAT strain. The slopes of the regression lines are 2.96 in the parental and 2.4 in the selected lines respectively (Table 3). In the KGB strain, the LT50 values were 33.5 min and 50.8 min and the corresponding slopes of the regression lines were 3.26 and 1.7 in the parental and selected populations respectively (Table 3). The change in slope of regression lines between KGB selected and parental indicates increased resistance in the selected population.

Cross-resistance to permethrin

The populations of *An. arabiensis* DDT selected in both MAT and KGB parental strain were also tested against the diagnostic dosage of 0.75% permethrin to check for cross resistance or increased tolerance (Figure 3). Significantly more mosquitoes were knocked down by permethrin at 30 min, 40 min, ($P < 0.001$) and at 50 min ($P = 0.072$) in the parental line than in the DDT selected

Table 3. Relative susceptibility of DDT (4%) based on time mortality relationships tested against parental and selected lines of *An. arabiensis* MAT and KGB strains.

Strain	Line	Sample	No. tested	LT50 (min)	CI	LT90	Slope	RR	X^2
MAT	Parental	F20	355	23.4	(20.3 - 26.1)	63.40	2.9 ± 0.3	1	0.703
	Selected	F20	290	33.2	(29.2 - 37.7)	88..96	2.40 ± 0.3	1.4	1.301
KGB	Parental	F20	360	35.1	(28.2 - 37 .3)	95.06	3.26 ± 0.5	1.5	0.821
	Selected	F20	581	50.8	(44.1 - 66.6)	139.56	1.70 ± 0.4	2.2	1.25

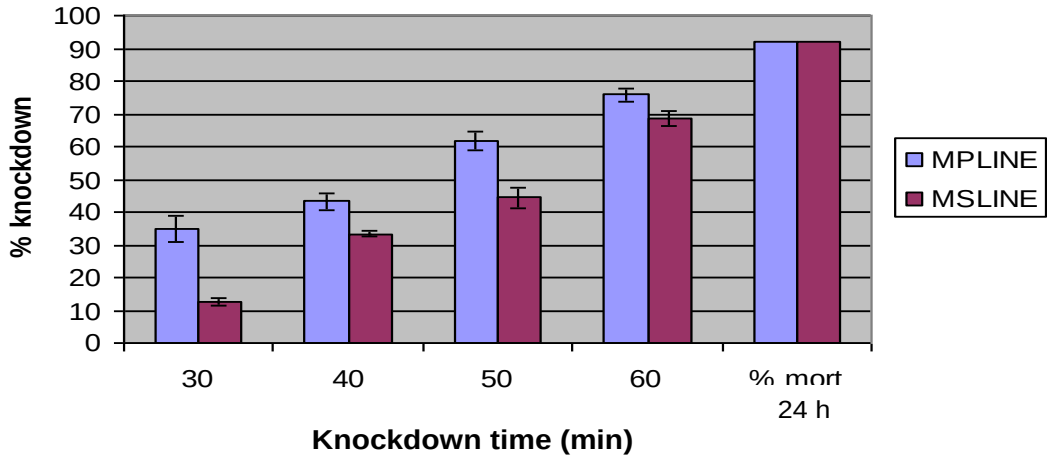

Figure 3. Percentage knockdown and percentage mortality of 1 to 3 day-old adult F18 on 0.75% permethrin. MPLINE (MAT parental line) (n = 261) and F18 MSLINE (MAT selected line) (n = 144) during 30 – 60 minute exposure to 0 .75% permethrin and 24 h after exposure respectively.

Table 4. Comparisons of geometric means (with 95% confidence limits) for GST activity in *An. arabiensis* MAT and KGB strains.

Variable	Line	N	Mean (95% confidence interval)		Test statistic	p-value
GSTact	Mparental	59	0.139	(0.120 - 0.161)		
	Mselected	61	0.236	(0.209 - 0.267)	Mp vs Ms	< 0.001
	KGBPG6	60	0.183	(0.170 - 0.198)		-
	KGBSG9	70	0.221	(0.202 - 0.241)	Kgbg6 vs g9	0.014
	KGBSG15	92	0.189	(0.173 - 0.205)	Kgbg6 vs g15	0.022

Test statistics: GSTact:- F(118) = 5.579 MAT p < 0.001, F(2,219) = 5.002 KGB p = 0.014. KGBPG6 denotes KGB parental generation 6, KGBSG9, KGB selected generation 9, KGBSG15, KGB selected generation 15.

population but both showed > 97% mortality at 24 h after exposure (Figure 3).

Biochemical assays

Glutathione S – transferase activity

The geometric mean GST activity was significantly higher ($p < 0.001$, 0.014) in populations under selection pressure than in the parental (unselected) populations in *An. arabiensis* MAT and the ninth generation KGB strains respectively (Table 4).

Esterase activity

The geometric mean values of α-esterase and β-esterase

Table 5. Comparisons of geometric means (with confidence limits) for alpha and beta esterase activities between adults sampled from the parental and selected lines of *An*. *arabiensis* MAT and KGB strains.

Variable	Line	N	Mean (95% confidence interval)		p-value
Alfact	Mparental	59	0.000399	(0.000363 – 0.000438)	
	Mselected	61	0.000653	(0.000551 – 0.000774)	< 0.001
	KGBPG6	60	0.000700	(0.000655 – 0.000749)	-
	KGBSG9	69	0.000862	(0.000791 – 0.000940)	0.004
	KGBSG15	107	0.000583	(0.000539 – 0.000630)	<0.001
Betact	Mparental	59	0.000271	(0.000247 – 0.000297)	
	Mselected	61	0.000447	(0.000373 – 000535)	<0.001
	KGBPG6	60	0.000536	(0.000504 – 0.000571)	
	KGBSG9	70	0.000670	(0.000615 – 0.000729)	< 0.001
	KGBSG15	92	0.000423	(0.000392 – 0.000456)	<0.001

Table 6. (Geometric) mean monooxygenase activity levels (with 95% confidence intervals) in *An*. *arabiensis* MAT and KGB strains.

Strain	Line	N	Mean (95% confidence interval)		p-value
MAT	Parental	38	0.000192	(0.000163 – 000227)	-
	Selected	32	0.000410	(0.000300 – 0.000559)	0.001
KGB	KGBGP6	72	0.000171	(0.000158 – 0.000184)	-
	KGBSG9	114	0.000186	(0.000173 – 0.000201)	0.148
	KGBSG15	89	0.000161	(0.000150 – 0.000172)	<0.677

activities were significantly higher ($p < 0.001$) in the selected than in the parental lines of the MAT strains. In the KGB strain, the esterase activities were higher in the KGBS9 compared to KGBP6 ($p < 0.001$). However, the activities in KGBSG15 were lower than that in KGPG6 ($P < 0.004$) (Table 5).

Monooxygenases activity

The geometric means of monooxygenases in the selected and parental lines in the *An. arabiensis* MAT and KGB strains were not significantly different ($p = 0.148$) (Table 6).

DISCUSSION

The results of this study indicate that a low level of physiological resistance to DDT in *An. arabiensis* is developed under selection pressure in the laboratory. The LT50 and LT90 values of DDT increased significantly over 15 generations of selection pressure in both MAT and KGB *An. arabiensis* strains. The LT50 and LT90 recorded for the twentieth generation of KGB selected line are similar to the values reported for the DDT resistant field populations of *An. gambiae* sl (Tarig et al*.,* 2018). In the selection of *An. arabienisis* MAT strain, high variation characterised the mortality values during the first four generations. This might have been due to the error made initially in the selection process by putting the survivors from the first two selection experiments back in the same cage with the parental colony. Mating between the two populations might have resulted in dilution of the selected resistant genotypes; therefore making the population more susceptible to DDT as was earlier hypothesized (Prasittisuk and Curtis, 1982). Alternatively, it has been suggested that high variation in mortalities is perhaps typical of populations in early stages of selective pressure (Theeraphap et al., 2002).

The general patterns of the selection for DDT resistance are similar in both strains. This observation is typical of most laboratory regimes which tend to select within existing phenotypic distributions often at 80 – 90%

mortality in order to provide survivors for the next generation (Martins et al., 2012; Roush and McKenzie, 1987). The dosage for selection was closely controlled between 30 to 45 min and 30 – 60 min for the MAT and KGB respectively to permit discrimination among similar genotypes within the physiological distribution of phenotypes (Roush and McKenzie, 1987). However, in previous similar studies, 3 laboratory colonies G1, SENN and MBN of *An*. *arabiensis* have been selected for resistance to DDT at higher doses and adults were reported to have survived exposure to DDT for 8 h (Hemingway, 1981; Matambo et al., 2007). This suggests that the KGB and the MAT strains were at comparatively low level of DDT resistance. Theoretically, a susceptible colony comprising of totally susceptible individuals will produce the highest slope for a regression line of dose – response data. With selective pressure from the exposures to insecticides, a population will become heterozygous for resistant genotypes and as the frequency of resistant genotypes increases, the slope of the regression line will shift to the right (Brown and Brogdon, 1976). There was a shift to the right in regression lines from dose response data for the populations under DDT selection in both the MAT and KGB strains and the slopes of regression lines based on the data from these experiments continuously declined over time in the two strains. This suggests that the resistance to DDT in the selected populations was not due to vigour tolerance but reflects true physiological resistance (Oliver and Brooke, 2014; Brown and Brogdon, 1976).

Evidence for cross resistance to permethrin was observed in the DDT – selected colony of *An*. *arabiensis* MAT strain Various previous studies have shown some evidence for resistance to permethrin in colonies of *An. arabiensis* strains selected for DDT resistance (Nardini et al., 2013; Matambo et al., 2007). In addition, cross resistance between pyrethroids and DDT has been reported in natural populations of *An. arabiensis* (Abdulla et al., 2008), *An. gambiae* (Matawo et al., 2015), and *An. funestus* (Tchouakui et al., 2019). The similar mode of action of DDT and pyrethroids can result in cross-resistance if the mechanism is due to kdr mutations in the sodium ion channel target sites (Martinezz-Torre et al., 1998; Tene et al., 2013). The West African L1014F mutation has previously been reported in *An. arabiensis* from Sudan (Abdullah et al., 2008) and SENN-DDT resistant laboratory strain (Matambo et al., 2007), although in both the correlation between the L1014F genotype and DDT resistant genotype, it was not clear.

In this study, analysis of sequence data for the gene revealed absence of *kdr* mutations in both MAT and KGB colonies. Similarly, the kdr mutation has not been observed in the M form of *An. gambiae* ss and *An. arabiensis* despite high levels of resistance to pyrethroid and DDT although it was found in the S form (Diabete et al*.,* 2002). The kdr mutations have been documented in DDT resistant field populations of *An. coluzzi*, *An. gambiae* ss and *An. arabiensis* (Cisse et al., 2015). The combined effects of detoxifying enzymes and potential mutations have been associated with resistance to multiple insecticides in *An. funestus* and *An. arabiensis* (Menze et al*.,* 2016; Matawo et al., 2014) Nevertheless, the absence of the *kdr* mutations in the colonies of *An. arabiensis* studied here is not conclusive considering the low number of samples used in the assay.

The results of biochemical analysis have shown that more individuals with high GST activity are present in the selected than in the parental lines of both the MAT and KGB strains. The order of magnitude of change in GST activity observed in the selected populations of MAT and KGB strains is consistent with our recent report on involvement of the epsilon gste2 gene in DDT resistance in the colonies (Yayo et al., 2018, 2019). Previous studies have severally associated resistance to DDT with increased levels of GST activities in several species of mosquitoes including *An. subpictus* (Hemingway et al., 1991) and *An*. *gambiae* (Karunaratne et al*.,* 2018). Elevated esterase activity was also detected in the MAT populations under DDT selection compared to the unselected population.

The results in absolute unit for the alpha and beta esterase were similar to those of Hargreaves et al. (2003). Casimiro et al. (2006) found lower average esterase activities with the two substrates in DDT susceptible populations of *An. arabiensis* from Mozambique. The monooxygenase activity was low in the selected populations in KBG strain, suggesting that the p450 enzyme system may not be involved in DDT resistance in this strain. However, the monooxygenase activity was significantly higher in some individuals from selected line compared to the parental line in MAT strain, but the small sample size was low to derive a conclusion.

Conclusion

Two laboratory strains of *An. arabiensis* exposed to controlled doses of DDT have developed resistance to DDT and cross resistance to permethrin. Analyses of the detoxification enzymes have shown significantly high GST activity in the selected line of both strains. Preliminary investigations revealed absence of kdr suggesting the possible role GST-based DDT resistance mechanism in the colony.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison and optimization for DNA extraction of okra (*Abelmoschus esculentus* L. Moench)

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The extraction of high-quality DNA from okra (*Abelmoschus esculentus* L. Moench) is notoriously troublesome due to the high contents of polysaccharides, polyphenols, and different secondary metabolites. We have tested seven extraction buffers on silica dried okra leaves. Here, we describe a simple, rapid and modified procedure for high-quality DNA extraction from okra, which is amenable for downstream analyses. In contrast to Cetyl-trimethyl-ammonium bromide (CTAB) methods, the described procedure is rapid, omits the use of liquid nitrogen, phenol, PVP-10, and chloroform. It also uses inexpensive and less hazardous reagents and requires only ordinary laboratory equipment. The procedure employed a high concentration of Sodium dodecyl sulphate (SDS) to rid the problems associated with polysaccharides and polyphenols. The average yield was between 36 and 45 µg of total DNA from 90 mg of dried leaf weight. The DNA is adequate for molecular analysis of okra, such as genetic mapping or marker-assisted plant breeding. This protocol can be performed in as little as 3 h and may be adapted to high-throughput DNA isolation.

Key words: PVP-10, polyvinylpyrrolidone, non cetyltrimethylammonium bromide, okra, genomic DNA, Sodium dodecyl sulphate (SDS).

INTRODUCTION

Okra (*Abelmoschus esculentus* L. Moench), belonging to the Malvaceae family, is an edible vegetable species that is widely cultivated and distributed in the tropical, subtropical and warm temperate areas of the world (Kumar et al., 2013; Lamont, 1999; Diizyaman, 1997; Martin, 1982). The crop is believed to be originated in Ethiopia (Harlan, 1969). It is usually cultivated for its edible immature fruit in fresh or dried form. Nevertheless, the plant is also used as a source of protein (Gemede et al., 2015), fiber (Khan et al., 2017), biomass (Lee et al., 2018), oil (Wei et al., 2017), mucilage (Fronda et al., 2018), colorants (Waghela and Khan, 2018), traditional medicine (Dubey and Mishra, 2017), pharmaceuticals (Zhang et al., 2018) and as ornamental crop (Diizyaman, 1997).

Okra is a highly diversified crop (Akotkar et al., 2010). The wide economic importance of the plant is urging its improvement for different purposes. Thus, breeding elite varieties and developing different cultivars is needed. Even though okra has a wide range of importance and its

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center of origin and diversity is in Ethiopia (Harlan, 1969), molecular variability studies and cultivar improvement programs are still at their infancy.

For studying the molecular characteristics and genetics of okra, extraction of high-quality DNA in sufficient quantity is important. However, extraction of high quality and quantity DNA is often a limiting factor in okra (Porebski et al., 1997), since a large number of species from the Malvaceae family contain secondary metabolites like mucilage (Singh and Kumar, 2012) including alkaloids, phenolic compounds, gummy polysaccharides, terpenes and quinine (Ali et al., 2019; Amani et al., 2011; Porebski et al., 1997; Singh and Kumar, 2012).

During cell lysis, nucleic acids come into contact with these polysaccharides in the oxidized form. The polyphenols bind covalently and irreversibly to proteins and nucleic acids resulting in a brown gelatinous material (Agrawal et al., 2016). This reduces the yield and purity of DNA. Thus, the quality and quantity of DNA will interfere with the subsequent reactions such as PCR, gene cloning and restricted DNA digestion, and sequencing.

Several protocols have been developed for genomic DNA extraction for okra (Jeyaseelan et al., 2019; Seth et al., 2018; Singh and Kumar, 2012). However, these protocols involve the use of hazardous and technologically demanding chemicals like phenol, chloroform, and liquid nitrogen. These chemicals are not recommended in open laboratories with no specialized rooms and safety cabinets. For these reasons, an effective and appropriate protocol is needed for isolating genomic DNA for genotyping, PCR work and sequencing of okra with inexpensive resources while avoiding the health implications of phenol and chloroform (Mahuku, 2004).

The objective of this study was to develop a straightforward technique to isolate deoxyribonucleic acid, a way that eliminates the necessity to use phenol or chloroform to purify the DNA. The resulting SDS (Sodium dodecyl sulphate) protocol was used to isolate high quality genomic DNA subject to PCR analysis from different accession of okra with reduced cost and health concerns. The results will be used for further molecular studies. It also supports technology inaccessible countries to exclude the high cost and impact of phenol-chloroform, liquid nitrogen and enzymes like RNase.

MATERIALS AND METHODS

The DNA extraction process was carried out at the Plant Cell Laboratory, School of Plant and Horticultural Sciences, Hawassa University. Young and healthy leaves from forty-four okra accessions grown in the field were collected from the agricultural field of Hawassa University. The leaves were divided into two batches, one batch was dried with silica gel and the fresh samples were kept in plant collection bag for temporary use.

Solutions

Seven genomic DNA extraction buffers (KCL, CTAB, and SDS) were used with and without modifications in the buffer composition (Table 1).

Protocol for SDS based DNA extraction

1. Ninety (90) mg of silica dried okra leaf tissue were ground to a fine powder with the help of three metal tungsten carbide beads over the vortex mixer supported by micropipette tip based crushing of a sample in an Eppendorf tube.
2. Eight hundred (800) µl of cell lysis buffer (0.5% SDS (w/v) in 10XTE) was added to each tube followed by vortexing at high speed for approximately 2 min until the powder was fully hydrated and mixed with buffer.
3. Samples were incubated for 10 min at room temperature (RT).
4. This step was followed by precipitation of genomic DNA with 200 µl 3 M sodium acetate (pH 5.2) and mixed by inversion of tubes.
5. The mixture was incubated on ice for 5 min.
6. Samples were centrifuged at 16,000Xg for 5 min at RT to pellet the leaf material.
7. The liquid material was transferred carefully to an empty 1.5 ml centrifuge tube.
8. An equal volume of isopropanol was added to the supernatant and completely suspended by vortexing and inverting the tubes (approximately 20 s).
9. Samples were incubated for 15 min at RT by inverting tubes every three minutes by hand.
10. The samples were centrifuged at 16,000Xg for 3 min at RT followed by removal of supernatant with a pipette.
11. 500 µl of freshly prepared wash buffer (5 M NaCl and 95% ethanol) was added to each tube and completely suspended by vortexing the tubes (approximately 20 s).
12. The step was followed by centrifuging the sample at 16,000Xg for 3 min at RT to pellet the genomic DNA.
13. The last step was the removal of the supernatant and washing the pellet with 75% cold ethanol (4°C).
14. The pellet was allowed to dry at room temperature before elution with 60 µl of 1X TE buffer.
15. The DNA was stored temporarily at 4°C before checking its quality and quantity.

Protocol for KCl based DNA extraction

1. Ninety (90) mg of silica dried okra leaf tissue was weighted.
2. Sample was placed into a 1.5 ml tube.
3. 400 ul of DNA extraction buffer (1 M KCl, 100 mM Tris-HCl, 10 mM EDTA) was added to the tube.
4. Sample was crushed by the tip of the pipette inside the Eppendorf tube.
5. Sample was incubated for 30 min (~1 h) at 65°C in a water bath and consequently centrifuged for 10 min at 15000 rpm at 25°C.
6. 100 µl of isopropanol was added to a new 1.5 ml tube or 96-well plate while waiting.
7. 100 µl of supernatant was transferred into the tube prepared in step 6.
8. Samples were mixed by pipetting or inverting tube.
9. The mixture was centrifuged at maximum speed (>2800 rpm) for 30 min at 4°C.
10. The supernatant was discarded by inverting the tube.
11. 150 µl of 70% ethanol was added to wash the pellet.
12. The mixture was centrifuged at maximum speed (>2800 rpm) for 15 min at 4°C.
13. The supernatant was discarded by inverting the tube.
14. The tube was placed upside down for about 10 (~30) min to dry.
15. 30 µl of 1xTE were added to elute the DNA.
16. Samples were kept at 4°C for at least one day to elute well.

Table 1. Composition and concentration of chemicals used for DNA extraction buffers.

Chemical	Lysis Buffer (LB)						
	LB1	LB2	LB3	LB4	LB5	LB6	LB7
NaCl (M)	-	1.4	1.4	-	-	1.4	-
KCl (M)	1	-	-	-	1	-	-
EDTA (mM)	10	20	20	-	10	20	-
Tris-HCl (mM)	100	100	100	-	100	100	-
CTAB (%)	-	2	2	-	-	2%	-
PVP (%)	-	1%	-	-	-	-	-
SDS (%)	-	-	-	0.5	2	-	0.5
βMercapto ethanol (µl)	-	-	2	-	-	-	-
Extraction method	KCL	CTAB	CTAB	SDS	KCL	CTAB	SDS
Modifications	-	1% PVP	2%ß mercapto ethanol	-	2% SDS	1% PVP	Isopropanol precipitation
Reference	Unpublished	Allen et al. (2006)	Allen et al. (2006)	Xia et al. (2019)	-	Unpublished	Xia et al. (2019)

Protocol for CTAB based DNA extraction

Protocol for CTAB was followed according to Devi et al. (2013).

Gel electrophoresis

2 µl of eluted DNA were subjected to electrophoresis on a 0.8 mg/ml ethidium bromide stained 1% (w/v) agarose (Thermo Scientific, USA) gel in 1XTris-EDTA (TE) buffer. The agarose gel was documented on a GelDoc (BIO-RAD, USA).

Qualitative and quantitative analyses of the isolated DNA

The yield and purity of extracted DNA was assessed using Nano-Drop 2000 (Thermo-Scientific, USA), by measuring the concentration and UV absorption ratios at A260/280 and A260/230. Agarose gel electrophoresis was performed to determine the level of DNA degradation and contamination by RNA.

PCR amplification confirmation

PCR was carried out in a thermal cycler (BIO-RAD, USA) to amplify the specific DNA sequence, in a reaction volume of 10 µl containing PCR buffer (10 mMTris-HCl, 50 mMKCl), 50 ng of the DNA, 1.5 mM $MgCl_2$, 0.5 units of Taq polymerase (Fermentas, USA), 0.1 mM of dNTPs (Fermentas, USA), 10 pM of each ribosomal gene-specific 18S forward (5`-AACGGCTACCACATCCAAGG-3`) and reverse (5`-TCATTACTCCGATCCCGAAG-3`) primers. The amplification conditions were: initial denaturation for 5 min at 95°C, followed by 30 cycles of 45 s denaturation at 94°C, 45 s annealing at 55°C and 45 s extensions at 72°C. Final extension step was at 72°C for 10 min.

Two SSR primers (ST-1) synthesized by (Metabion international AG, Germany) were used for detecting the functionality of the extracted DNA by this developed protocol. The PCR reaction (10 µl volume) contained about 50 ng of genomic DNA, 0.1 µl of blend taq polymerase (2.5 u/µl, Top-Bio s.r.o., Czech Republic), 2 µl 10× PCR reaction buffer, 0.1 µl $MgCl_2$ (25 mM), 0.2 µl dNTP mixture (2.0 mM), 0.2 µl each primer (25 µM), and dH_2O up to 10 µl. Amplification was carried out in a T100 BIO-RAD thermal cycler. Cycling conditions consisted of 5 min initial denaturation at 95°C, followed by 1 min denaturing at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C repeated for 35 cycles and 5 min extension at 72°C. PCR products were subsequently separated by electrophoresis on 2% agarose gel at 70 V for 40 min, which was then stained with ethidium bromide (0.5 mg/ml) and photographed as described above by using 1.5 µl of 1 kb DNA ladder.

Data analysis

Data were analyzed on the DNA concentration and on the 260/280 and 260/230 ratios by agricolae package (de Mendiburu, 2020) using the R software version 3.5 (R Core Team, 2018). Tukey's pairwise comparisons with the confidence interval of 95% were used to compare the concentration between the extraction methods.

RESULTS AND DISCUSSION

This method largely follows those already developed for other plant species in the major steps, such as cell disruption, DNA extraction and precipitation (Kalbande et al., 2016; Paterson et al., 1993); however, there were modifications to the composition to the lysis buffer, which were intended to overcome the issues of high secondary metabolite content in the plant tissue. Specifically, a phenol-binding reagent (SDS) and NaCl were used to remove polyphenols and polysaccharides, respectively.

In addition, to reduce the cost and processing time of the procedure, all buffers were prepared from chemicals available in local stores.

The analyzed results for the ratio of UV absorption $A_{260}/_{280}$ and $A_{260}/_{230}$ and the concentration of DNA extracted by the different modified KCl, CTAB and SDS methods are presented in a box plot (Figures 1 and 2). The mean concentration and quality of DNA is presented in Table 2. Comparison of quality and quantity values (Table 2) indicated that there is no significant difference between different accessions on: the concentration ($F = 0.34$, $df = 3$, $P = 0.08$), $A_{260/280}$ ratio ($F = 0.26$, $df = 3$, $P = 0.86$), and the $A_{260/230}$ ratio ($F = 0.18$, $df = 3$, $P = 0.91$). This contradicts with the principle that plant species belonging to the same or related genera can exhibit

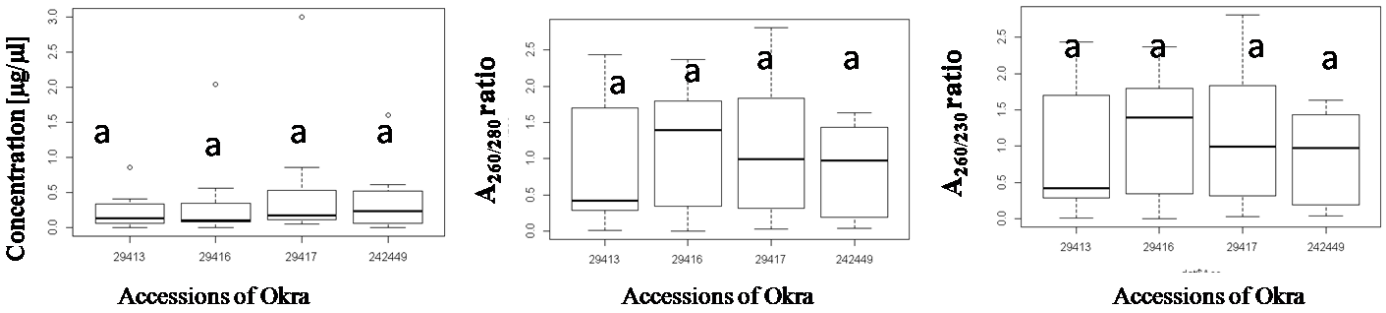

Figure 1. Box plot of DNA concentration and OD value from four Accessions of Okra using the seven different extraction buffers.

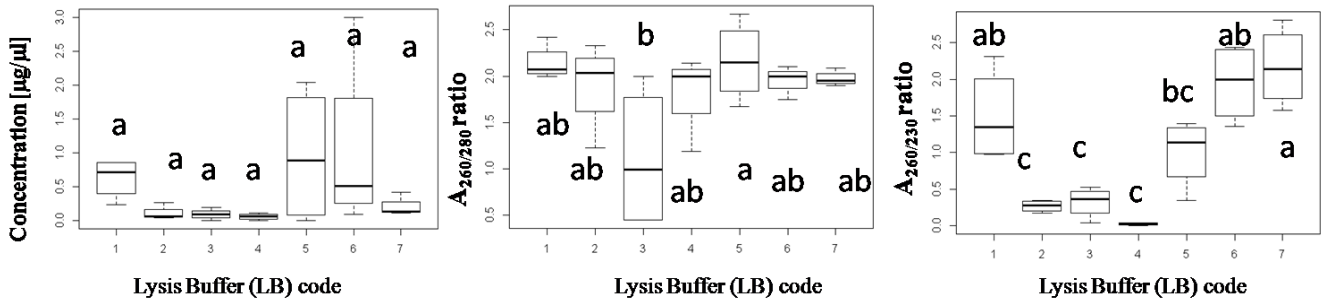

Figure 2. Box plot of DNA concentration and OD value of okra DNA extracted using the seven different extraction buffers.

Table 2. Analysis of variance on the concentration, 260/280 and 260/230 ratios of the seven extraction buffers used in the experiment.

Parameter	Lysis buffer			Accessions		
	Concentration (µg/µl)	$A_{260/280}$ ratio	$A_{260/230}$ ratio	Concentration (µg/µl)	$A_{260/280}$ ratio	$A_{260/230}$ ratio
DF	6	6	6	3	3	3
Sum of square	4.34	3.03	17.87	0.53	0.21	0.49
Mean square	0.72	0.51	2.98	0.18	0.07	0.16
CV	148.68	22.82	41.24	166.35	28.14	91.04
MSD	1.58	1.04	1.04	1.12	0.81	1.44
F value	1.72	2.78	16.44	0.34	0.26	0.18
Pr (>F)	0.17	0.04	0	0.8	0.86	0.91
Significance	NS	*	***	NS	NS	NS

NS – not significant, * - significant at P ≤ 0.05, *** - significant at P ≤ 0.001.

enormous variability in their biochemical composition and this heterogeneity may not permit optimal DNA yields from one isolation protocol and this leads to the development of different isolation protocol even for closely related genera. However, significantly different results were obtained comparing the different lysis buffers (Table 2) for $A_{260}/_{280}$ratio (F = 2.78, df = 6, P = 0.04) and for $A_{260}/_{230}$ratio (F = 16.44, df = 6, P = 0.00). Intactness and quality difference of the DNA extracts using the different lysis buffers is shown in Figure 3. The assessment of the purity of the DNA is confirmed by the $A_{260}/_{280}$ ratio. For a 'pure' nucleic acid, this value commonly resides in the range of 1.8 to 2.0 (Sambrook et al., 1989). The $A_{260}/_{280}$ ratios below approximately 1.3 and above 2.3 are indicators of poor quality of the DNA (Seth et al., 2018). Samples with absorbance ratio at $A_{260}/_{280}$ greater than 2 indicate the presence of carbohydrates and other secondary metabolites (Wilson and Walker, 2010). Higher values of absorbance ratios are evidence of contamination by phenols while lower values indicate the presence of proteins since proteins absorb light at a wavelength of 280 nm (Wilson and Walker, 2010). The presence of RNA in the sample has been also shown to increase the $A_{260}/_{280}$ ratio. RNA contamination can be

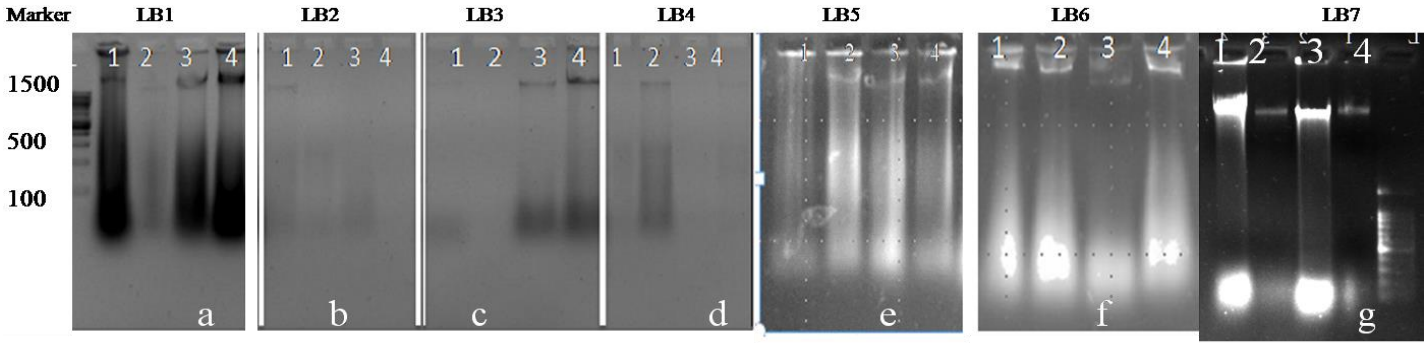

Figure 3. Electrophoresis of Okra DNA on 1% agarose gel. From a to g is the different extraction buffer used as indicated in Table 1. Lanes 1 to 4 indicates the different Okra accessions used for the experiment (1=29416; 2= 29417; 3= 242449; 4= 29413). The first and last lane is the 1.5 Kbp and 100 bp DNA ladder marker respectively.

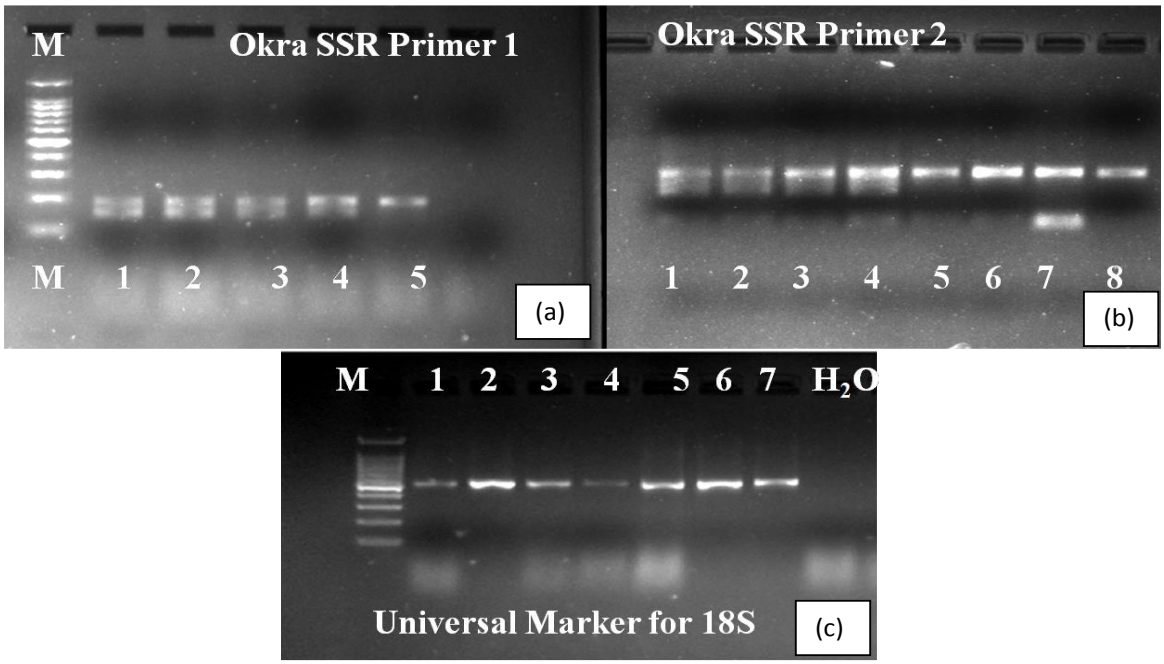

Figure 4. Two SSR and one Universal (18S) Primer patterns were used for the amplification of extracted Okra DNA using LB7 of representative Okra genotypes (a) Okra SSR marker 1 (b) Okra SSR marker 2 (c) Universal marker (18S) for the different samples of Okra (Lane 1 to to 8. M: 100 bp ladder).

confirmed or ruled out by agarose electrophoresis, in case of RNA being present in the sample; it has to be treated by RNase (Valledor et al., 2009).

In this experiment, the majority of the lysis buffers resulted in the formation of smear on the gel, which indicated the degradation of the DNA (Figure 3). However, lysis buffer 7 (LB7) formed a relatively better band with less smear, which also hints at absence of RNA contamination (Figure 3). The absorbance ratio of $A_{260/280}$ for the four tested samples ranged between 1.9 and 2.0. The values of all samples were within the accepted range, indicating a low level of contamination (ST-2). Similar work was also conducted for extracting DNA from samples with a high amount of polysaccharides and mucilage using SDS to replace CTAB extraction buffer (Sharma et al., 2018). SDS is an anionic detergent for cell and nucleus lysis to release ribonucleic and deoxyribonucleic acids by inhibiting the nucleases, ribonuclease (RNase) and deoxyribonuclease (DNase) activities (Farrell, 2011). The resulting DNA was further used for SSR marker-based analysis (Figure 4).

CTAB based DNA extraction method is the most commonly used technique for different crop species. However, in our current experiments CTAB was not satisfactory in terms of quality and quantity of DNA with/without modification and with modification of CTAB

extraction components like PVP, β- mercaptoethanol (Figure 3). The extraction of DNA from fresh okra samples using CTAB resulted in a thick and sticky substance, which could not be pipetted out of the Eppendorf tubes. This can be explained by the presence of polysaccharides in the DNA sample which form a highly viscous solution (Jeyaseelan et al., 2019). Mucilage is a highly viscous secondary metabolite composed of a polar polymer of glycoprotein that can co-precipitate with DNA and inhibits the action of Taq polymerase (Menu et al., 2018). Polysaccharides are problematic as they make the DNA unreliable during pipetting and hinder the activity of polymerases and restriction endonucleases (Kumar et al., 2018).

Conclusion

This study compared and optimized quality and speedy protocol which will be used for routine DNA isolation from okra (*A. esculentus* L. Moench) and is amenable for marker-assisted breeding, and high-throughput applications. In addition, this protocol may be used for other plant species that are recalcitrant to other methods due to their high levels of polysaccharides and polyphenols.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Supplementary Tables (ST)

Table S1. List of primers used for PCR amplification of the genomic DNA extracted by the improved protocol.

Primer	Primer Sequence	Type	Expected Size	PIC	TM (°C)
18S-FW	AACGGCTACCACATCCAAGG	Universal	500	-	55
18S-RW	TCATTACTCCGATCCCGAAG	Universal			55
Ok1-FW	TCATGTCTTTCCACTCAACA	SSR	194	0.58	54
Ok1-RW	CCAAACAAAATATGCCTCTC	SSR			54
Ok3-FW	AACACATCCTCATCCTCATC	SSR	203	0.73	56
Ok3-RW	ACCGGAAGCTATTTACATGA	SSR			54

Table S2. DNA yields, 260/280 and 260/230 ratios obtained from (Lysis buffer 7 (LB-7) for the 44 Okra genotypes.

Accession	Concentration (µg/µl)	$A_{260/280}$ Ratio	$A_{260/230}$ Ratio
24213	0.23	1.8	0.9
29408	0.77	2	1.2
29409	0.45	1.9	1
29410	0.75	1.9	0.9
29411	0.53	1.7	0.8
29412	1.13	2	1.4
29413	0.57	2	1.1
29414	2.22	2.1	1.5
29415	1.09	2	1.3
29416	1.18	2.1	1.5
29417	0.88	1.8	0.9
240201	2.13	2.2	1.7
92203	0.9	2	1.5
240203	0.64	2	1.4
240204	0.59	2	1.3
240207	0.91	2.1	1.5
240209	1.1	2.1	1.5
240583	1.27	2.2	1.7
240585	1.35	2.2	1.9
240586	1.31	2.1	1.7
240587	0.31	2.1	2.1
240591	0.34	2.1	1
240592	0.34	2	1.4
240599	0.32	1.8	1.3
240600	0.43	1.8	0.8
240601	0.59	2.1	1.6
240602	0.32	1.9	1.1
240609	0.53	1.9	1.4
240615	0.68	2	1.6
240784	0.54	2.1	1.9
240786	1.94	2.1	1.7
242433	0.34	2.1	1.8
242443	0.71	2.2	1.8
242444	0.57	2.1	1.5
242445	0.56	2.1	1.5
242448	0.58	2.2	2
242449	0.6	2.2	1.9

Table S2. Contd.

New	**0.5**	**2.1**	**1.6**
242450	0.26	2	1.6
242451	0.52	2	1.5
245161	0.38	1.9	1
245162	0.47	2.1	1.4
Local	0.47	2	1.3
New	0.98	2.2	2
Improved	0.47	2.1	1.5

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Lactic acid bacteria fermentation of coconut milk and its effect on the nutritional, phytochemical, antibacterial and sensory properties of virgin coconut oil produced

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Coconut oil has profound health benefits but the high content of fatty acids is a concern to many consumers, as several processing methods have failed to produce oil with considerable change in fatty acid content. In this study, selected lactic acid bacteria including *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Leuconostoc mesenteroides* and *Enterococcus faecium* were used to ferment coconut milk for production of virgin coconut oil. Fermentation was carried out for 48 h, after which the milk was processed through heating to produce coconut oil. Phytochemical content, proximate composition, determination of steroids and free fatty acid content of the oil and antibacterial activity of the oil against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were carried out using standard analytical and laboratory procedures. The results obtained in this study indicate that the control and LAB fermented oil had similar composition of phytochemicals; steroids, anthraquinone and glycosides were present in all the virgin coconut oil produced; however, reduced moisture content, lower levels of ester and free fatty acid was observed and higher quantity of protein and ash were obtained for coconut oil fermented with LAB compared to the control. The virgin coconut oil produced using the starter culture also had higher acceptability ($P<0.05$) compared to the spontaneously fermented oil, as the milk fermented with *Lactobacillus plantarum* had the highest acceptability value. There was no significant difference in the antibacterial activity of the virgin coconut oil produced against the test organisms. This study indicates that free fatty acid and other undesirable constituents in coconut oil can be reduced using lactic acid bacteria as starter culture, hence, increasing the acceptability of the product.

Key words: Lactic acid bacteria, coconut milk, fermentation, coconut oil.

INTRODUCTION

Coconut oil is extensively used for food, industrial applications, health promotion and disease prevention (Manisha and Shyamapada, 2011). Natural fermentation is a method where less processing conditions are involved. In the natural fermentation process, extracted milk from wet coconut was allowed for microbial

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fermentation (Divina, 2002). Lactic acid bacteria (LAB) for a long time have been applied as starter cultures in fermented foods and beverages because they can improve nutritional, organoleptic, technological and shelf-life characteristics. LAB can also produce ethanol, bacteriocins, aroma components, exopolysaccharides and some enzymes (De Vuyst and Leroy, 2004).

Lactic acid bacteria play a key role in the VCO fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety. Fermentation is one of the most ancient and most important food processing technologies. Fermentation is a relatively efficient, low energy preservation process, which increases the shelf life and decreases the need for refrigeration or other forms of food preservation technology. LABs are considered Generally Recognized as Safe (GRAS). LABs produce antimicrobial agents including organic acids, hydrogen peroxides and bacteriocin. LAB has been known for many years and play important role in the production of a variety of fermented foods. Health benefits of LAB are known to give positive influence in the gastrointestinal of humans (Hafidh et al., 2010). Lactic Acid Bacteria (LAB) constitute part of the autochibonous microbiota of many types of foods. They are defined as a clutter of lactic acid-producing Low G+C%, non-spore forming, Gram-positive rods and cocci and catalase-negative which share many bio-chemical, physiological and genetic properties (Abriouel et al., 2004). This group of bacteria has a particular interest for food industries due to their technological properties, being often used as starter cultures to produce fermented products.Virgin coconut oil (VCO) seemed to be the purest form of coconut oil, water white in color, contains natural vitamin E and with very low free fatty acid content and low peroxide value. It has a mild to intense fresh coconut aroma. VCO may be defined as the naturally processed (Nour et al., 2009), chemically-free and additive-free product from fresh coconut meat or its derivative (coconut milk and coconut milk residue) which has not undergone any further chemical processing after extraction (Marina et al., 2009).

This study therefore aimed at using selected lactic acid bacteria as starter culture for fermentation of coconut milk and evaluating the effect on quality attributes of the coconut oil produced.

MATERIALS AND METHODS

Coconut sample

Randomly selected uniformly sized, 12 months old (matured) nuts were collected from local commercial market.

Source of indicator organisms

Staphylococcus aureus and *Pseudomonas aeruginosa* were isolated from spoilt coconut fruits.

Microbial culture

Pure culture of five lactic acid bacteria isolated from fresh fruits possessing antibacterial activity and safe to be used as probiotics were used as seed culture in different vessels; one milliliter of 10^5MacFarland of each of the bacteria was introduced into 250 ml of coconut milk for fermentation.

Coconut milk extraction

Coconuts were broken and solid endosperm was collected, testa was removed by using kitchen peeler, white coconut balls were disintegrated into small pieces and grinded with 1:2 ratio of water for 10 min. Ground mass was transferred to the muslin cloth, pressed manually for coconut milk extraction; the same process was repeated twice and coconut milk was pooled up.

Phytochemical and physicochemical composition

The saponification, iodine and acid value were determined according to the titre metric method of Pearson (1981) while peroxide value was evaluated according to AOAC (1984).

Proximate composition

Proximate composition of the virgin coconut oil: Moisture content, ash, carbohydrate, crude protein, fat and crude fiber were determined according to AOAC (1984).

Determination of antibacterial activity

The indicator organisms were streaked on sterile molten Mueller Hinton agar, paper discs were impregnated with oil and Vancomycin paper disc was used as control following the method of Nguyen et al. (2017).

Statistical analysis

The means of the results were evaluated using analysis of variance (ANOVA), and the Tukey test was used to compare differences ($p < 0.05$) among the technological, physicochemical, microbiological and sensory evaluations. Statistical Analysis System (SAS, 1999) was used.

RESULTS AND DISCUSSION

Phytochemical screening of the virgin coconut oil (Table 1) reveals that all the samples contain steroids, terpenoids, cardiac glycosides while alkaloids, flavonoids, saponins and tannins were absent. Ghosh et al. (2014) detected terpenoid and steroids in virgin coconut oil and their report is similar to our study; however, tannins were present in their oils contrary to our observation in this study. The presence of these phytochemicals in coconut oil vary as different researchers differ in their reports.

Though there is dearth of information on induced

Table 1. Phytochemical composition of coconut oil produced using lactic acid bacteria starter.

S/N	Starter fermented samples	Alkaloids	Flavonoids	Saponins	Tannins	Anthraquinnones	Terpenoids	Steroids	Cardiac glycosides
1	IB	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
2	C_{19}	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
3	C_{17}	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
4	C_{26}	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
5	C_{12}	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve
6	CCO	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve

H: Hot; C=Cold; -ve= Negative; +ve =positive; MC=Moisture content; CP=Crude protein; Crude Fiber; CCO= Control; IB=sample fermented with *Leuconostoc mesenteroides*; C_{12} = sample fermented with *Lactobacillus plantarum*in hot water extraction; C_{17}= sample fermented with *L. pentosus*; C_{19}= sample fermented with *L. plantarum*in cold water extraction; C_{26}= samples fermented with *Enterococcus faecium*.

Table 2. Physicochemical quality of coconut oil produced using lactic acid bacteria starter.

Starter fermented samples	Saponification value	Iodine value	Ester value
IB	444.6	19.98	424.62
C_{19}	402.9	20.62	382.28
C_{26}	468.4	19.99	434.62
C_{12}	420.2	20. 53	387.28
C_{17}	403.4	20. 42	384.28
CCO	472. 4	20.62	451.78

H: Hot; C=Cold; MC=Moisture content; CP=Crude protein; Crude fiber; CCO= Control; IB=sample fermented with *Leuconostoc mesenteroides*; C_{12} = sample fermented with *Lactobacillus plantarum* in hot water extraction; C_{17}= sample fermented with *.pentosus*; C_{19}= sample fermented with *L. plantarum*in cold water extraction; C_{26}= samples fermented with *Enterococcus faecium.*

Table 3. Physical parameters of coconut oil produced using lactic acid bacteria starter.

S/N	Starter fermented samples	Refractive index	pH value	Specific gravity
1	IB	1.654	3.86	0.9130
2	C_{19}	1.657	3.28	0.9140
3	C_{17}	1.654	3.42	0.0.925
4	C_{26}	1.655	3.43	0.8990
5	C_{12}	1.660	3.26	0.8890
6	CCO	1.652	3.69	0.9230

CCO= Control; IB=sample fermented with*Leuconostoc mesenteroides*; C_{12} = sample fermented with *Lactobacillus plantarum*in hot water extraction; C_{17}= sample fermented with *L. pentosus*; C_{19}= sample fermented with *L. plantarum* in cold water extraction; C_{26}= samples fermented with *Enterococcus faecium.*

fermentation of coconut milk by lactic acid bacteria, the result obtained in this study as regards the physicochemical quality of the oil meets international codex standard and agrees with the report of Satheesh and Prasad (2012) that produced coconut oil using lyophilized culture of *Lactobacillus plantarum.* The moisture content of the coconut oil produced in this study ranges between 0.11 to 0.40; the international standard is 0.1 -0.5, all the LAB strains used produce coconut oil with very low moisture content (0.11 -0.20), and it is believed that moisture plays a major role in the shelf life of oils as it influences its rancidity.Our report is not similar to what was reported by Satheesh and Prasad (2012) that used innovative wet process for production of coconut oil. Physiochemical property of the coconut oil (Table 2) such as iodine value and ester value ranges between 382.28 to 451.78, the refractive index ranges between 1.65 to 1.66, the specific gravity is between 0.89 to 0.93 and pH values ranged from 3.26 to 3.86 (Table 3). Further, a significant difference exist between the specific gravity of virgin coconut oil produced using *L. plantarum* along with hot and cold extraction method, as the result of the quality parameters of the VCO produced in this study meets quality standard and is similar to earlier report of

Table 4. Proximate composition of coconut oil produced using lactic acid bacteria starter.

S/N	Sample ID	% MC	% CP	%CF	%Ash
1	CCO	0.30	0.71	98.91	0.09
2	IB	0.21	1.08	98.61	0.21
3	C_{12}	0.29	1.75	97.85	0.30
4	C_{17}	0.20	1.35	98.46	0.20
5	C_{19}	0.13	1.03	98.71	0.13
6	C_{26}	0.39	1.06	97.61	0.20

MC=Moisture content; CP=Crude protein; Crude Fiber; CCO = Control; IB=sample fermented with *Leuconostoc mesenteroides*; C_{12} = sample fermented with *Lactobacillus plantarum* in hot water extraction; C_{17} = sample fermented with *L. pentosus*; C_{19} = sample fermented with *L. plantarum* in cold water extraction; C_{26} = samples fermented with *Enterococcus faecium*.

Table 5. Sensory evaluation of starter fermented coconut oil.

Sample	Taste	Aroma	Colour	Flavor	Overall acceptability
CCO	4.4^{a} ±0.50	4.0^{a}±0.99	4.55^{b}±0.76	4.35^{a}±0.49	4.33^{a} ±0.50
IB	4.05^{a}±0.83	4.10^{a}±0.91	4.75^{ab}±0.55	4.60^{a}±0.50	4.49^{a} ±0.75
C_{12}	4.05^{a}±0.95	4.05^{a}±0.95	4.70^{ab}±0.66	4.55^{a}±0.51	4.32^{a} ±0.51
C_{17}	4.00^{a}±0.97	4.30^{a}±0.73	4.80^{ab}±0.37	4.55^{a}±0.51	4.40^{a} ±0.85
C_{19}	4.30^{a}±0.73	4.25^{a}±0.85	4.85^{ab}±0.37	4.55^{a}±0.51	4.41^{a}±0.76
C_{26}	4.00^{a}±0.65	4.00^{a}±0.80	4.75^{ab}±0.44	4.75^{ab}±0.44	4.30a±0.54

The values represent mean± standard deviation. Also, values with the same superscript are not significantly different; CCO=control spontaneous fermentation.

Table 6. Antibacterial activity of starter fermented coconut oil against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Sample	*Staphylococcus aureus*		*Pseudomonas aeruginosa*	
	Zone of inhibition (mm)	Vancomycin	Zone of inhibition (mm)	Vancomycin
C_{12}	4	5	4	5
C_{17}	2.5	5	3	5
C_{26}	4	4	2.5	7
IB	2	4	3	5
C_{19}	3	5	5	6
CCO	3	4	2	5

CCO= Control; IB=samples fermented with *Leuconostoc mesenteroides*; C_{12} = samples fermented with *Lactobacillus plantarum* in hot water extraction; C_{17}= samples fermented with *L. pentosus*; C_{19}= samples fermented with *L. plantarum* in cold water extraction; C_{26}= samples fermented with *Enterococcus faecium.*

Thanuja et al. (2016).

Quantitative determination of steroids in the virgin coconut oil produced shows that the control has high percentage of steroids and free fatty acid compared to the oil fermented with specific starter culture of selected. There is significant difference.in the quantity of protein present in LAB fermented coconut milk (1.07 to 1.76) and spontaneously fermented coconut (1.06) for coconut oil production. Higher protein content was obtained compared to the control samples; and the moisture content of the oil was lower in all LAB produced coconut oil except the oil produced using *Leuconostoc mesenteroides*, reduced moisture and ash content compared to the control (Table 4).

All the oil samples have similar general acceptability in terms of flavor and color. Oil produced using *L. plantarum* had a higher acceptability in terms of taste while the aroma of the oil produced with *L. mesenteroides* had the highest acceptability (Table 5). The zone of inhibition produced by using coconut oil in agar well diffusion method as antibacterial against *S. aureus* (Table 6) is similar to the standard antibiotics.

Conclusions

Based on our findings, it can therefore be said that fermentation of coconut milk with LAB as starters for

production of virgin coconut oil (VCO) be encouraged as the VCO produced had better nutritive and sensory properties, a prominent attribute of foods fermented with lactic acid bacteria. Also, the fatty acid and moisture content of the oil produced is significantly lower compared to the control, a property most desired in edible oils.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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TetraSOD® activates the antioxidant response pathway in human cells: An *in vitro* approach

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The combined action of both endogenous and exogenous factors stimulates the production of free radicals and reactive oxygen species (ROS) in cells. They are neutralized by an elaborate antioxidant defense system, superoxide dismutase (SOD) being the first line of enzymes involved in ROS scavenging. However, when ROS production cannot be counteracted by cellular antioxidant mechanisms oxidative stress happens, inducing damages in proteins, lipids and DNA. It had been suggested that antioxidant supplementation may help to reduce oxidative stress. TetraSOD® is a unique marine healthy and functional ingredient that is produced under patent-protected technology, which exhibits a balanced nutritional composition and a significantly high SOD activity (>30,000 U/g). In this work, the antioxidant bioactivity of TetraSOD® in muscle cells (Normal Human Skeletal Muscle Myoblasts, HSMMs) was examined using an *in vitro* approach. Three different doses of SOD activity in TetraSOD® extracts were tested in cells: 30, 150 and 1500 U/ml. A statistically significant induction of the primary antioxidant enzyme activities, SOD, glutathione peroxidase (GPx), and catalase (CAT) was observed at the two higher doses in relation to the control. These results were further related to the expression of a selected set of genes involved in response against oxidative stress. A significant up-regulation of *SOD1*, *SOD2*, *GPx1*, *CAT*, *NRF2* and *HMOX1* was detected after TetraSOD® treatment with regard to the control, exhibiting different patterns in response to the applied dose to cells. Overall, the results obtained in this study might represent an important contribution to the understanding of the molecular basis underlying the potential cytoprotective effects induced by TetraSOD® consumption.

Key words: TetraSOD®, *Tetraselmis chuii*, oxidative stress, antioxidant, gene expression, enzyme activity, SOD, NRF2.

INTRODUCTION

Cells produce reactive oxygen species (ROS) as a consequence of the own metabolic processes, the superoxide anion being the precursor of all other ROS (Romao, 2015). The three main *in vivo* sources for superoxide anion are the mitochondrial respiratory chain complexes, the nicotinamide adenine dinucleotide phosphate-oxidase, and xanthine oxidases. ROS can provoke damages in proteins, lipids and DNA when

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antioxidant capabilities are overwhelmed by the burden of ROS, a state known as “oxidative stress” (Tebay et al., 2015). To detoxify ROS cells have developed an elaborate antioxidant defense system in which superoxide dismutase (SOD) is the first line of enzymes involved in scavenging of ROS. This enzyme reduces superoxide anion to hydrogen peroxide (H_2O_2), and then glutathione peroxidase (GPx) and catalase (CAT) convert H_2O_2 into H_2O and O_2 (Ighodaro and Akinloye, 2018).

Occurrence of oxidative stress as a consequence of both endogenous metabolism and exogenous factors (diet, pollution, UV radiation, smoking, mental stress…) is considered to be involved in a number of diseases such as cancer, atherosclerosis, diabetes, chronic inflammation, and neurodegenerative and cognitive disorders (Fiedor and Burda, 2014). In this scenario, dietary factors seem to play a pivotal role in the regulation of the oxidant status as a diet low in antioxidants contributes to oxidative stress (Décordé et al., 2010). A wide range of studies have examined the beneficial effects of antioxidant supplementation to reduce oxidative stress; particularly, SOD is probably one of the antioxidant proteins most extensively addressed (Carillon et al., 2013a; Romao, 2015; Stephenie et al., 2020). In this regard, an increase in endogenous antioxidant enzymes (not only SOD but also GPx and CAT) has been observed after SOD supplementation both at protein and activity levels, supporting the hypothesis that exogenous SOD could induce endogenous antioxidant enzymes (Vouldoukis et al., 2004; Nelson et al., 2006; Carillon et al., 2013a, b, 2014; Romao, 2015). However, the molecular mechanisms underlying such induction processes have not been elucidated yet.

TetraSOD® is a unique marine healthy and functional ingredient that is grown under patent-protected technology (Unamunzaga and Mantecón, 2015). In addition to its exhibited high SOD activity (>30,000 U/g), it shows a balanced nutritional composition containing essential fatty acids, vitamins, minerals, amino acids and pigments. With the aim to understand the physiological bases of the protective health effects of TetraSOD®, *in vitro* testing of bioactivity was assessed in human cells as a first step in the elucidation of mechanisms explaining such protection. Thus, the effect of TetraSOD® in the three main antioxidant enzyme activities (SOD, GPx and CAT) in Normal Human Skeletal Muscle Myoblasts (HSMMs) was determined. Moreover, to gain additional insights on the TetraSOD® effect at molecular level, the expression profile of a selected set of genes encoding for proteins involved in protection against oxidative stress was analyzed including: (i) the three distinct identified isoforms of SOD in mammals. Two of the isoforms have Cu and Zn in the catalytic center and exhibit different localization, being intracellular (Cu,Zn-SOD or SOD1) or extracellular (EC-SOD or SOD3). The last isoform has Mn as a cofactor (Mn-SOD or SOD2) and is localized to mitochondria (Zelko et al., 2002; Sheng et al., 2014); (ii) glutathione peroxidases, which couple oxidation of glutathione with detoxification of H_2O_2. The family comprises up to eight isoforms in mammals. Among them, both GPx1 and GPx4 are ubiquitous as have been identified in the cytosol, nucleus and mitochondria of the cells, and also exhibit a wide tissue distribution (Margis et al., 2008; Espinosa-Diez et al., 2015); (iii) the human catalase, which is an heme-containing peroxidase that forms a tetramer composed of four subunits, each one divided in four domains, and plays a predominant role in controlling the concentration of H_2O_2 (Putnam et al., 2000; Goyal and Basak, 2010); (iv) the heme oxygenase-1 (HMOX1) inducible phase II detoxifying enzyme, which is considered an important component involved in the adaptive and protective response to multiple oxidative insults (Solano and Arck, 2015; Liao et al., 2018); (v) the nuclear factor erythroid 2-related factor 2 (NRF2), which is a transcription factor that under physiological conditions is sequestered by binding to Kelch-like ECH associated protein (KEAP1), thus inhibiting translocation of NRF2 into the nucleus. When KEAP1 changes its conformation due to interaction with different inducers, NRF2 is released and translocated to the nucleus, regulating the cytoprotective response to oxidative stress through transcriptional activation of phase II detoxifying and antioxidant enzymes (Dinkova-Kostova and Talalay, 2008; Tkachev et al., 2011). Main results of this research are presented here in after.

MATERIALS AND METHODS

Microalgae biomass (TetraSOD®)

TetraSOD® was produced by Fitoplancton Marino, S.L. using a patent protected technology. It corresponds to a lyophilized powder of the microalgae *Tetraselmis chuii (T. chuii)* strain CCFM 03 (belonging to the culture collection of Fitoplancton Marino, S.L.), which is grown in controlled outdoor close photobioreactors (proprietary process) under photoautotrophic conditions ensuring high SOD activity (>30,000 U/g) (patent protected).

Extract preparation and SOD activity determination in TetraSOD®

Extract was prepared from TetraSOD® using a proprietary methodology. A 10% dry weight solution in phosphate buffer (KH_2PO_4 200 mM pH=7.8) was disrupted using a high pressure homogenizer. Cell debris was removed by centrifugation at 20,000 g for 15 min at 4ºC, and the supernatant was recovered and stored at -80ºC until freeze-drying. An aliquot was first removed to determine SOD activity, which was quantified using the SOD Assay Kit-WST (Dojindo) according to the Technical Manual. The inhibition rate was determined by a kinetic method using a time range between 2 and 10 min, in which a very high linearity was observed (R^2>0.99).

Cell culture and incubation with TetraSOD® extract

Normal human skeletal muscle myoblasts (HSMMs, Clonetics™)

Table 1. List of primers used in this study.

Gene symbol	Gene name	Primer sequences	Amplicon length (bp)	Optimal Ta (ºC)	Reference
SOD1	Superoxide dismutase 1	F: 5'-GGATGAAGAGAGGCATGTTGGA-3' R: 5'-TAGACACATCGGCCACACCAT-3'	71	56.9	Letsiou et al. (2017)
SOD2	Superoxide dismutase 2	F: 5'-ACAAGTTTAAGGAGAAGCTGACGG-3' R: 5'-CTCCCACACATCAATCCCCAG-3'	173	60.2	This work
SOD3	Superoxide dismutase 3	F: 5'-TGGATCCGAGACATGTACGCCAA-3' R: 5'-GGAAGAGGACGACGCCGGTCA-3'	154	66.5	This work
GPx1	Glutathione peroxidase 1	F: 5'-CGATGTTGCCTGGAACTTTGAG-3' R: 5'-ATGTCAATGGTCTGGAAGCGG-3'	90	60.5	Letsiou et al. (2017)
GPx4	Glutathione peroxidase 4	F: 5'-CGCTGTGGAAGTGGATGAAGAT-3' R: 5'-AGCCGTTCTTGTCGATGAGGA-3'	99	59.5	Letsiou et al. (2017)
CAT	Catalase	F: 5'-TGCTGAGAAGCCTAAGAATGCG-3' R: 5'-ACAGATTTGCCTTCTCCCTTGC-3'	80	57.9	Letsiou et al. (2017)
HMOX1	Heme oxigenase 1	F: 5'-CGATGCACACCACATGACCGACC-3' R: 5'-CGCCTGCTTCATCGCCTTGACC-3'	122	60.0	This work
NRF2	Nuclear factor erythroid 2-related factor 2	F: 5'-CCAAAGAGCAGTTCAATGAAGC-3' R: 5'-GCAGCCACTTTATTCTTACCCC-3'	76	55.3	Letsiou et al. (2017)
ACTB	Beta actin	F: 5'-CTGTCCACCTTCCAGCAGATGT-3' R: 5'-AGCATTTGCGGTGGACGAT-3'	79	60.9	Letsiou et al. (2017)

were purchased from Lonza (Walkersville, USA) and cultured according to instructions provided by the manufacturer. They were grown in SkGM™-2 BulletKit™ medium in a humidified incubator at 37°C with a 5% CO_2 atmosphere. Cells were subcultured when they were 50 to 70% confluent and contained many mitotic figures throughout the flask.

Cells were incubated in 6-well plates for 24 h with TetraSOD® extract prepared at different dilutions (2 ml total volume per well) containing 30, 150 and 1500 U/ml of culture media. As a control treatment, phosphate buffer was employed. Afterwards, cells were rinsed twice with PBS and directly processed for enzyme activities determination or RNA isolation as described subsequently. The experiment was repeated twice, and in each one, treatment was performed in triplicate.

SOD, GPx and CAT activity determination in HSMMs cells

Cells were detached by gentle treatment with Trypsin/EDTA solution provided with the ReagentPack™ Subculture Reagents (Clonetics™). After cells were released, trypsin was neutralized with Trypsin Neutralizing Solution (Clonetics™). Thereafter, cells were washed twice in ice cold phosphate buffer by centrifugation at 220 g for 5 min at 4ºC. Then cells were resuspended in phosphate buffer containing 1% peroxide-free Triton X-100. Complete lysis was achieved by softly pipetting up and down several times. Finally, cell lysate was centrifuged at 20,000 g for 10 min at 4ºC, and the supernatant was recovered and immediately stored at -80ºC until use.

SOD activity was quantified using the SOD Assay Kit-WST (Dojindo) according to the Technical Manual as previously indicated. GPx activity (nmol/min/ml) was quantified using the Glutathione peroxidase activity kit (Enzo) as described in the manual provided by the manufacturer. CAT activity (nmol/min/ml) was determined using the Catalase Assay Kit (Cayman Chemical) following the kit booklet. In all instances, activity values were transformed to percentages in relation to the Control (without TetraSOD® incubation).

RNA isolation and gene expression analysis

Total RNA was isolated from HSMMs cells using the NucleoSpin® RNA Kit (Macherey-Nagel) in accordance with the manufacturer's protocols. A double on column DNase I treatment for 30 min was performed to avoid amplification of genomic DNA. Quality of RNA samples was first checked in agarose gels, and then A_{260}/A_{280} and A_{260}/A_{230} ratios (>2.1) were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). Total RNA (1 µg) from each sample was reverse-transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

Real-time analysis was carried out on a CFX96™ Real-Time System (Bio-Rad) using specific primers for *SOD1*, *SOD2*, *SOD3*, *GPx1*, *GPx4*, *CAT*, *HMOX1* and *NRF2* (Table 1). Real-time reactions were accomplished in a 10 µL volume containing cDNA generated from 10 ng of original RNA template, 300 nM each of specific forward and reverse primers, and 5 µl of iQ™ SYBR® Green Supermix (Bio-Rad). The amplification protocol included an initial 7 min denaturation and enzyme activation step at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each PCR assay was done in duplicate. For normalization of cDNA loading, all samples were run in parallel with the reference gene beta actin (*ACTB*) as previously reported (Letsiou et al., 2017). Relative mRNA expression was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Results were expressed as mean ± SD. Significant differences were determined with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. These analyses were performed

Table 2. Relative enzyme activities after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (U per ml of culture media) for 24 h.

Activity	Control	30 U/ml	150 U/ml	1500 U/ml
SOD	100.5 ± 3.1^{a}	121.7 ± 6.2^{ab}	133.8 ± 6.3^{b}	136.7 ± 6.2^{b}
GPx	105.7 ± 6.3^{a}	113.2 ± 9.8^{ab}	183.0 ± 12.0^{c}	172.2 ± 15.6^{bc}
CAT	100.8 ± 6.9^{a}	131.3 ± 9.1^{ab}	142.2 ± 9.9^{b}	137.3 ± 10.0^{b}

Values are expressed as percentages of activity with regard to the Control (100%). Data were expressed as the mean fold change (mean ± SD, n = 6; two assays in triplicate) from the calibrator group (Control). Different superscript letters denote statistically significant differences among doses ($p<0.05$) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

using the Prism *v*6 software (GraphPad Software). Significance was accepted at $p<0.05$.

RESULTS

Antioxidant enzyme activities after TetraSOD® treatment

The effect of TetraSOD® on the three antioxidant enzyme activities SOD, GPx and CAT was analyzed. In all instances, the lowest dose of TetraSOD® did not provoke a statistically significant change in activity when compared to the Control. However, in both 150 and 1500 U/ml treatments a significant increase was detected in the three activities. In SOD, the increase ranged around 34% in 150 U/ml and 37% in 1500 U/ml in relation to the control. In GPx activity, the highest variation was observed, ranging between 83 and 72% at 150 and 1500 U/ml, respectively. And in CAT, the increase in activity values ranged between 42 and 37% at 150 and 1500 U/ml, respectively (Table 2).

TetraSOD® induces up-regulation of genes involved in antioxidant response

Incubation of HSMMs with TetraSOD® extract up-regulated some of the most relevant genes involved in cellular antioxidant response (Figure 1). With 30 U/ml, a significant 4.49-fold increase in *SOD1* mRNAs with regard to the control was observed. At 150 U/ml it increased to 4.32-fold, and it was 3.80-fold higher (although not significant with regard to the control) at 1500 U/ml. No significant differences were found between any of the TetraSOD® treatments. Incubation of HSMMs cells with TetraSOD® also up-regulated *SOD2* at the three concentrations tested, with an increase in transcript amounts ranging between 2.02-fold at 1500 U/ml and 2.14-fold at 150 U/ml, with no statistically significant differences between treatments. In contrast to *SOD1* and *SOD2*, *SOD3* did not exhibit differences in mRNA abundance between any of the treatments and the control.

Moreover, TetraSOD® induced a significant up-regulation of *GPx1* at both 150 U/ml (2.14-fold higher than Control) and 1500 U/ml (2.23-fold increase). At 30 U/ml, a 1.85-fold increase was measured, although it was not statistically significant in relation to the control. In contrast, no significant changes were detected in *GPx4* between any of the treatments and the control.

In addition, treatment of HSMMs cells with both 150 and 1500 U/ml of TetraSOD® provoked a significant increase in mRNA levels of *CAT* with regard to the control (1.47-fold and 1.55-fold, respectively). A non-statistically significant 1.17-fold increase in transcripts was observed at 30 U/ml, which was significant when compared to 1500 U/ml treatment.

Finally, when HMMS cells were incubated with TetraSOD® at 30 U/ml, no significant changes in *NRF2* transcripts were detected. In contrast, a significant increase in mRNAs was measured both at 150 (2.05-fold) and 1500 (2.04-fold) U/ml. Moreover, a significant up-regulation of *HMOX1* was observed after treatment with 30 and 150 U/ml, exhibiting 1.19- and 1.54-fold higher transcript amounts than the Control, respectively. However, no significant differences were observed at the highest TetraSOD® dose.

DISCUSSION

Oxidative stress occurs when molecular defense systems are not able to counteract oxidation caused by both endogenous processes (such as mitochondrial metabolism) and/or exogenous factors including chemicals, pollution or diet. Hence, it reflects an imbalance of any biological system to detoxify ROS or repair resulting damages in cell components (Tebay et al., 2015). In the present study, the antioxidant effect of TetraSOD® using an *in vitro* approach in HSMMs cells was investigated, and the underlying molecular mechanisms of antioxidant protection were further explored. In this regard, a significant induction in the activity of the antioxidant enzymes SOD, GPx and CAT was observed after TetraSOD® treatment. On this matter, a literature search revealed a wide range of compounds of different nature (peptides, chemical reagents, natural

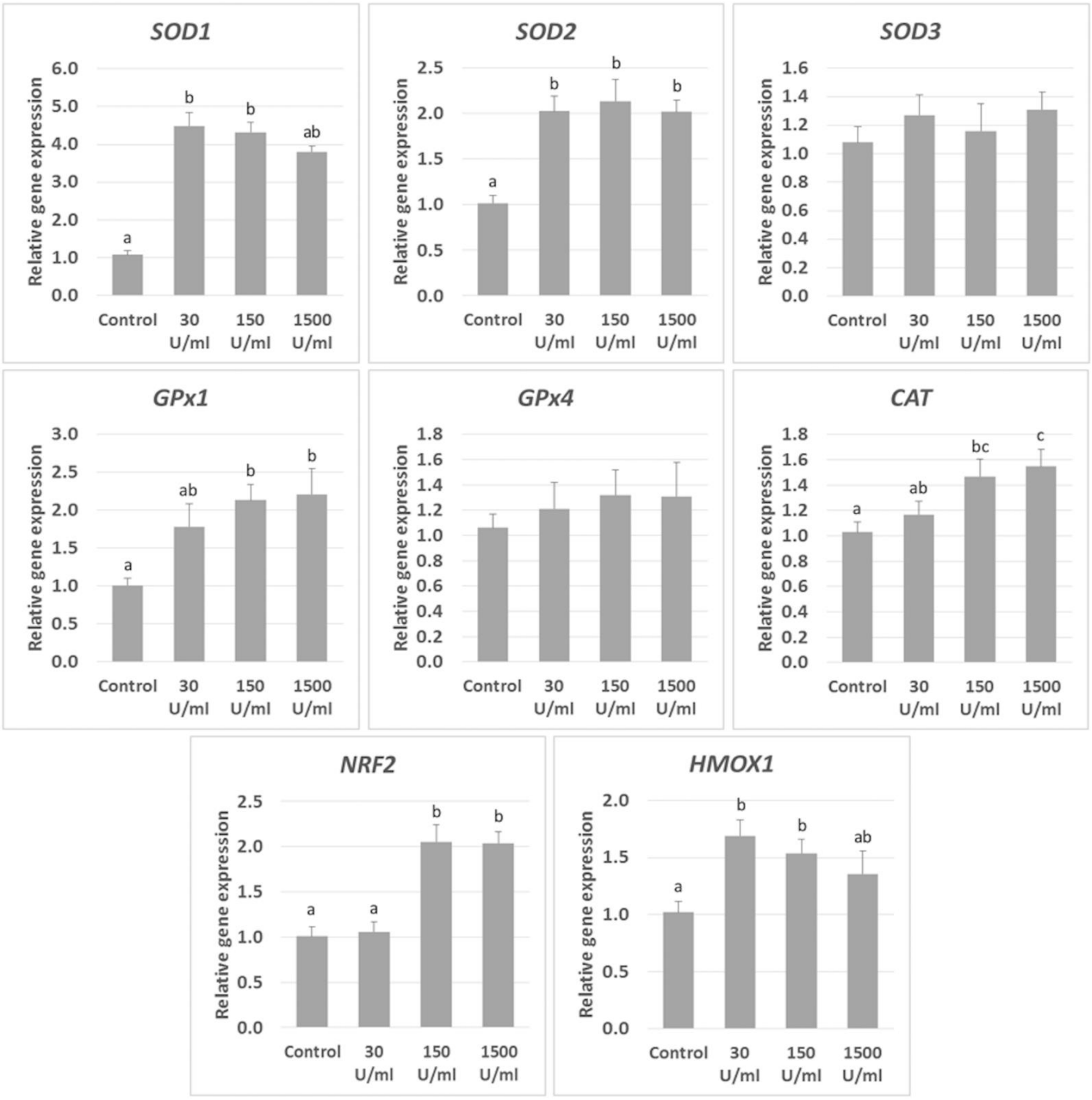

Figure 1. Relative gene expression levels of *SOD1*, *SOD2, SOD3*, *GPx1*, *GPx4*, *CAT*, *NRF2* and *HMOX1* after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (U per ml of culture media) for 24 h. Expression values were normalized to those of *ACTB*. Data were expressed as the mean fold change (mean ± SD, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

extracts…) able to induce activity of antioxidant enzymes in different cell systems. For instance, pretreatment of hippocampus cells with the cocaine- and amphetamine-regulated transcript (CART) peptide prevented the oxidative damage induced by amyloid-beta microinjection through a mechanism involving up-regulation of SOD activity (Jiao et al., 2018). An increase in not only SOD but also GPx activity has been observed in response to

oxidative damage induced by UV-A on normal human dermal fibroblasts after treatment with polypeptide from *Chlamys farreri* (Han et al., 2004). SOD activity was also up-regulated in human skin fibroblasts treated with the flavonoid baicalin in response to UV-A radiation (Zhou et al., 2012). Similar effects have been reported regarding SOD and CAT activity in human umbilical vein endothelial cells pretreated with pterostilbene (an homologous derivative of resveratrol) and further cultured under oxidative stress induced by uraemic serum (Chen et al., 2018). In addition, pretreatment of human HepG2 cells with the alkaloid epigoitrin increased SOD and GPx activity after exposure to H_2O_2 (Huo et al., 2017). All these responses found in cells have been associated to a general activation effect of antioxidant systems, in a complex scenario in which a battery of cytoprotective/defensive proteins including SOD, CAT and GPx are being up-regulated by the different compounds and treatments.

The analysis of transcriptional response of genes encoding the antioxidant enzymes and the further regulation of polypeptide translation has been less addressed. Unravelling this link is a key factor in order to understand the molecular mechanisms of cellular response against oxidative stress. For instance, a coordinated response in the induction of SOD, CAT and GPx activity, protein quantities and transcript amounts of *CAT*, *SOD1* and *GPx1* has been observed in HepG2 cells pretreated with the anthocyanin pelargonidin and then subjected to oxidative stress with citrinin (Babu et al., 2017). Similarly, human luteinized granulosa cells under oxidative stress induced by both H_2O_2 and dimethylfumarates exhibited a significant increase in *SOD1* and *CAT* mRNAs and their respective polypeptides (Akino et al., 2018). In this work, a parallel and positive response in enzyme activities and transcripts for SOD (*SOD1* and *SOD2*), CAT and GPx (*GPx1*) encoding genes in HSMMs cells as a consequence of TetraSOD® treatment was also found. Thus, the present results unravel the molecular basis of the cytoprotective effect of TetraSOD® in relation to the primary antioxidant enzymes.

In an attempt to go deeper in the knowledge of basal transcriptional regulation of antioxidant enzyme-encoding genes, the response to TetraSOD® treatment of genes *NRF2* and *HMOX-1* was then studied. NRF2 is a transcription factor widely expressed in eukaryotic cells which is known to activate antioxidant defenses. Actually, a number of reports have demonstrated that it is induced by antioxidant and chemopreventive compounds, which in turn regulates the expression of genes encoding both primary enzymes (that is, SOD, GPx, and CAT) and inducible phase II detoxifying enzymes such as HMOX1 (Krajka-Kuźniak et al., 2015; Fetoni et al., 2015; Tebay et al., 2015; Babu et al., 2017; Bahar et al., 2017; Hong et al., 2017; Liu et al., 2017; Teixeira et al., 2017; Chen et al., 2018; Wang et al., 2018). The results have demonstrated up-regulation of both *NRF2* and *HMOX1* expression when HSMMs cells are incubated with TetraSOD®, which strongly suggests the participation of the NRF2/HMOX1 pathway in the increased expression of *SOD*, *GPx* and *CAT* genes, and hence in the protective antioxidant status induced by TetraSOD®. Additional regulation at translational level of primary antioxidant enzymes has not been addressed in this work, and hence it cannot be ruled out in the absence of more data.

Cellular protection against oxidative damage involves two types of antioxidants: “direct” antioxidants, which can undergo redox reactions and scavenge ROS intermediates, and “indirect” antioxidants, which are inducers of cytoprotective proteins (Dinkova-Kostova and Talalay, 2008). TetraSOD® exhibits a significantly high SOD activity (as well as GPx and CAT activities; not shown). The action of these enzymes might contribute to scavenging of extracellular ROS owing to their own antioxidant activities. But in addition, TetraSOD® treatment induces a key set of genes belonging to the antioxidant machinery present in eukaryotic cells. It has been suggested that the antioxidant actions observed after consumption of a SOD-enriched product elaborated from melon might be triggered by a cascade of events possibly involving the NRF2 pathway (Carillon et al., 2013a, b, 2014), but no further experimental supporting data could be provided. The present results with TetraSOD® fully agree with that hypothesis and represent an important contribution to the understanding of the more complex physiological protective and preventive effects derived from TetraSOD® consumption. However, further research will be necessary to unravel the whole mechanisms rendering such positive effects *in vivo*.

CONFLICTS OF INTEREST

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Potential applications of enzymes on the extraction of vitexin from dried Mas Cotek leaves

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Mas Cotek or *Ficus deltoidea* var. *deltoidea* leaves extracts contain various beneficial properties such as antidiabetic, antihypoglycemic and antioxidant activities, which can be enzymatically extracted in water. Vitexin is one of the antioxidants and remarkable compound of flavone C-glycosides found in Mas Cotek leaves. Cellulase is one of the essential enzymes applied to hydrolyze cellulose into fermentable sugars and therefore, this enzyme was used to extract vitexin in this study. Single parameter approach was used to determine factors influencing the enzymatic-mediated extraction. These factors were enzyme concentration, sample-to-water ratio, temperature, incubation time and agitation. The maximum yield of vitexin found was 0.547% under the optimum conditions: sample-to-water ratio 1:10 g/mL, enzyme concentration 0.4%, incubation time of 240 min, temperature of 50 ± 0.02°C and stirring speed of 200 rpm. The highest amount of reducing sugar obtained was 4.039 mg/mL with the use of 0.4% cellulose. A correlation was also observed between the yield of vitexin and reducing sugars.

Key words: Cellulose, enzymatic assisted extraction, vitexin, reducing sugars, cellulose, Mas Cotek, *Ficus deltoidea* var. *deltoidea*, antioxidants.

INTRODUCTION

Mas Cotek or *Ficus deltoidea* var. *deltoidea* leaves extracts contain various beneficial properties such as anti-osteoporotic properties, anti-bacterial activity (Abdullah et al., 2018), and antidiabetic activity (Misbah et al., 2013; Papitha et al., 2018). Also, Mas Cotek extracts are found to be natural anti-oxidants (Sin et al., 2017). However, Mas cotek extraction is a challenge as it has low efficiency of aqueous extraction. In order to overcome the low efficiency of aqueous extraction process including potential loss of bioactive compounds, high energy and time consuming (Azwanida, 2015), the use of hydrolytic and cell-wall degrading enzymes as

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eco-friendly alternatives (Sharma and Gupta, 2006) are introduced which help to release the active compounds in the plant cell wall and increase the extraction yield. Rosenthal et al. (1996) had listed some advantages regarding the use of enzymatic extraction in terms of lowering the investment cost and energy requirement. The development of this method is also to fulfil the needs towards a clean environment especially the increasing awareness about volatile organic compounds (VOCs) caused by solvent emissions and safety concerns. In this study, cellulase enzyme was used to catalyse the hydrolysis of cell wall in Mas Cotek leaves.

Cellulase (EC 3.2.1.4) commonly used in the food industry to catalyse a wide variety of hydrolytic reactions and a high percentage act on cell wall polymers improving extraction yield of compounds, juices, oils and sugars (Salina et al., 2013). It is used for the extraction of oil from some oil-bearing seeds/fruits has been attempted in the laboratory or at the pilot industrial scale level (Domínguez et al., 1993; Rosenthal et al., 2001). The high cost of cellulase application had become the main limitation in the industrial processes, but with latest biotechnology advances it is already possible to obtain enzymatic formulations with lower costs and better quality (Zuniga et al., 2003).

Trichoderma reesei is a mesophilic and filamentous fungus. It is one of the good sources of cellulase enzymes. It produces 2-exo-β-D-glucanases, 5-endo-β-D-glucanases and 2-β-glucosidases. The whole hydrolysis process of cellulose can be divided into primary hydrolysis, that involves depolymerisation step by the action of endo-β-D-glucanase and exo-β-D-glucanase on the solid surface of substrate releasing soluble sugars and secondary hydrolysis, which involved the hydrolysis of cellobiose to glucose by β-glucosidase (Binod et al., 2011).

Cellulase is one of the thermostable enzyme that have several advantages including higher specific activity and higher stability which improve the overall hydrolytic performance (Viikari et al., 2007). As the catalytic efficiencies of enzymes improved, the cost of hydrolysis will be reduced eventually by enabling lower enzyme dosage (Dashtban et al., 2009). Based on the cellulase enzyme action, in this study, the enzymatic-assisted extraction was carried out to investigate the capability of cellulase enzyme in the optimum release of bioactive compounds (vitexin and isovitexin) from Mas Cotek leaves extracts, assisting the conventional aqueous extraction.

Antioxidant flavonoids like vitexin and isovitexin can be found in Agati et al. (2012), located in the intercellular plant cell. Chloroplast is a plant organelle that is responsible for the photosynthesis activity of a plant cell. It also carried out the synthesis of lipids and pigments and performed starch metabolism process. Beside the good health effects to human consumption, antioxidant flavonoids may effectively control the growth of plant cell and regulate the development of the whole plant and individual organs (Brunetti et al., 2013). As the leaves cell wall are predominantly made up of cellulose, hemicellulose and lignin, cellulase enzymes will assist in the digestion of lignin and bind to their cellulose substrate to generate glucose products in the extracts. The digestion of cell wall also released the intercellular components and active compounds into the extracts. Therefore, the objectives of this study were to investigate the effects of cellulase concentration and sample-to-water ratio on the extraction of vitexin compound from dried Mas Cotek leaves. Furthermore, to analyse active compounds and the amount of glucose released from the reaction of cellulase enzyme and plant cell activity were also determined.

MATERIALS AND METHODS

Chemicals and plant material preparation

Cellulase from *T. reesei* ATCC 26921 (lyophilized powder, ≥1 unit/mg solid) was purchased from Sigma-Aldrich (Missouri, USA). Vitexin standard (CODE: 1232 S) was ≥99% purity purchased from Extrasynthese (Genay Cedex, France). HPLC grade of methanol, formic acid (98 to 100%), acetonitrile, 50 mM acetate buffer and 0.1 M hydrochloric acid were purchased from Merck (Darmstadt, Germany). Dried leaves of Mas Cotek (*F. deltoidea* var. *deltoidea,* 4 kg) were obtained locally from Muadzam Shah, Pahang, Malaysia. Samples were further dried, cleaned and ground into small particles and kept in a freezer at -4°C until experiments were done. The uniformity of particle size was determined by sieving the leaves powder through an 850 µm (ASTM No. 20, 20 Mesh) standard sample sieve and a sieve shaker (Fritsch, Germany). Figure 1 shows the flow chart for the whole experimental work of the present study.

Cellulase-mediated extraction

Cellulase was quantified accurately and dispersed in deionized water to obtain enzyme solutions at various concentrations of 0.1 to 0.6% (g E/g substrate). Leaf powder samples of Mas Cotek were immersed in a mixture of deionized water and 50 mM acetate buffer (Merck, Darmstadt, Germany) at different sample-to-water ratio of 1:10, 1:20 and 1:30 g/mL (Badr and Sitohy, 1992; Ghose, 1987; Murashima et al., 2003). The solution pH was adjusted to 4.8 to 5.0 using certain amount of 0.1 M hydrochloric acid (Merck, Darmstadt, Germany). The mixture was put together in a 500 mL flask and incubated in a water bath (BS-21, Lab. Companion, Jeio Tech, Seoul, Korea) at 50°C with constant shaking at 200 rpm for 8 h. Cellulase solution was added after the sample solution achieved the optimum conditions for the cellulase to take action. A control sample (without cellulase addition) was prepared under the same condition in each ratio studied.

Five millilitres of samples were taken out in two separate test tubes at every 60 min time interval for active compound analysis and reducing sugar determination as will be described subsequently. Then, the samples taken were boiled at 100°C for 5 min to allow the denaturation of enzyme in the sample solution. After the enzyme deactivated, the extracts were centrifuged. Then, after 8 h extraction, the remaining solution in the beaker was filtered. The obtained residue was collected into another beaker

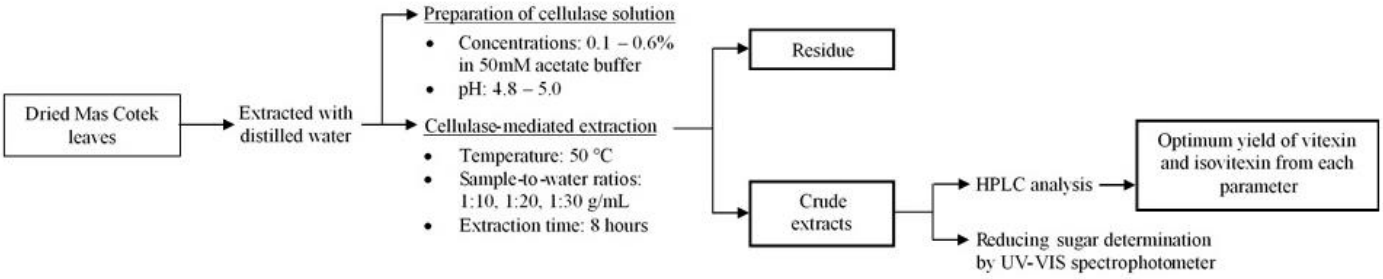

Figure 1. Flow chart for the whole experimental work of enzymatic-mediated extraction of vitexin from Mas Cotek leaves.

while the filtered solvent was discarded. The same proportion of distilled water was added into the sample residue according to the initial ratio. The aqueous extraction (without the enzyme) was done at 100°C for another 4 h. The extract was also sampled at every hour for further analysis. When the extraction process was accomplished, samples were cooled to room temperature and centrifuged and analysed using HPLC (Agilent, CA, USA) and UV-VIS Spectrophotometer (U-1800, Hitachi, Tokyo, Japan) for active compound and reducing sugar determination, respectively.

Reducing sugar determination

Glucose concentration was determined using 1% (w/v) solution of dinitrosalicyclic acid (DNS) based on Miller (1959) method. The sample taken every hour from enzymatic-mediated extraction of Mas Cotek leaves was centrifuged and appropriately diluted with deionized water up to 10 dilution factor. The diluted sample (1 mL) was mixed with 1 mL of DNS reagent and heated for 5 min on a boiling water bath. The absorbance of the cooled solution was measured at 575 nm against a blank that had been prepared using deionized water instead of the sample. The absorbance was converted to glucose concentration using a standard curve.

The standard curve had been prepared using glucose solutions of known concentrations (Shu et al., 2013; Zhang et al., 2009). One unit of cellulase will liberate 1.0 µmole of glucose from cellulose in 1 h at pH 5.0 and 37°C.

HPLC analysis

Samples of Mas Cotek leaves extracts from each extraction method were first analysed using HPLC once the extraction was done. Vitexin compound in this fine extract were identified and quantified by using a reversed-phase Phenomenex (Torrance, CA, USA) column (Prodigy, C_{18}, 5 µ, 250 × 4.60 mm i.d.). Calibrating curves of vitexin (ranging between 0.01 and 0.10 mg/ml) were prepared by diluting stock solution with the ultrapure water (Merck Millipore, USA). The HPLC consisted of a computer-controlled system with G1379A Degasser, G1311A QuatPump and G1321A fluorescence detector. Data acquisition was performed by Agilent ChemStation B.04.03 (Murashima et al., 2003). The column temperature was maintained at 25°C. Isocratic HPLC elution consisted of [A] 1.0% formic acid and water and [B] methanol were used. The mobile phases were filtered through a 0.2 µm nylon membrane and degassed using an ultrasonic bath (Crest Ultrasonics, Trenton, New Jersey) prior to use. The flow rate was set at 1.0 mL/min. Fluorescence detection was conducted at 335 nm wavelength. The concentration of compound in Mas Cotek leaves extracts were calculated by using the calibration equation of reference compounds. Based on the obtained concentration, actual percentage weight (%w/w) of vitexin was calculated by using the following formula (Equation 1):

Vitexin

$$\left(\%\frac{w}{w}\right)=\frac{[(Conc\,of\,compound\,from\,HPLC)(Volume\,of\,solution,L)(Dilution\,Factor)]}{(Weight\,of\,solute,mg)}\times 100 \quad (1)$$

Statistical analysis

The observations were replicated thrice for each parameter. Mean values were grouped and standard error (SE) was calculated. Statistical analysis was carried out using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Cellulase-mediated extraction

In the enzymatic-assisted or cellulase-assisted or mediated extraction (EnAE) of Mas Cotek leaves, 18 samples with replicate were prepared consisting of 6 different concentrations of hydrolytic enzyme (cellulase) ranging from 0.1 to 0.6% (g E/g substrate) in different sample-to-water ratios of 1:10, 1:20 and 1:30 g/mL. The solution pH was adjusted at 4.8 ± 0.05 and the solution temperature was kept constant at 50 ± 0.02°C throughout the experiment in a shaking water bath (200 rpm). Both pH and temperature were in the optimum conditions for cellulase enzyme to take action in the extraction process.

Effect of enzyme concentration and sample to water ratio

Cellulase enzyme concentration of 0.4% (g E/g substrate) gave the highest vitexin value of 0.547% with sample-to-water ratio of 1:10 g/mL at the 4th hour of extraction (Figure 2). This is where the enzyme activity reaches the peak and the catalytic efficiency is also the highest (Duan et al., 2015).

Compared to the highest vitexin yield obtained for control sample, the highest yield of vitexin from EnAE

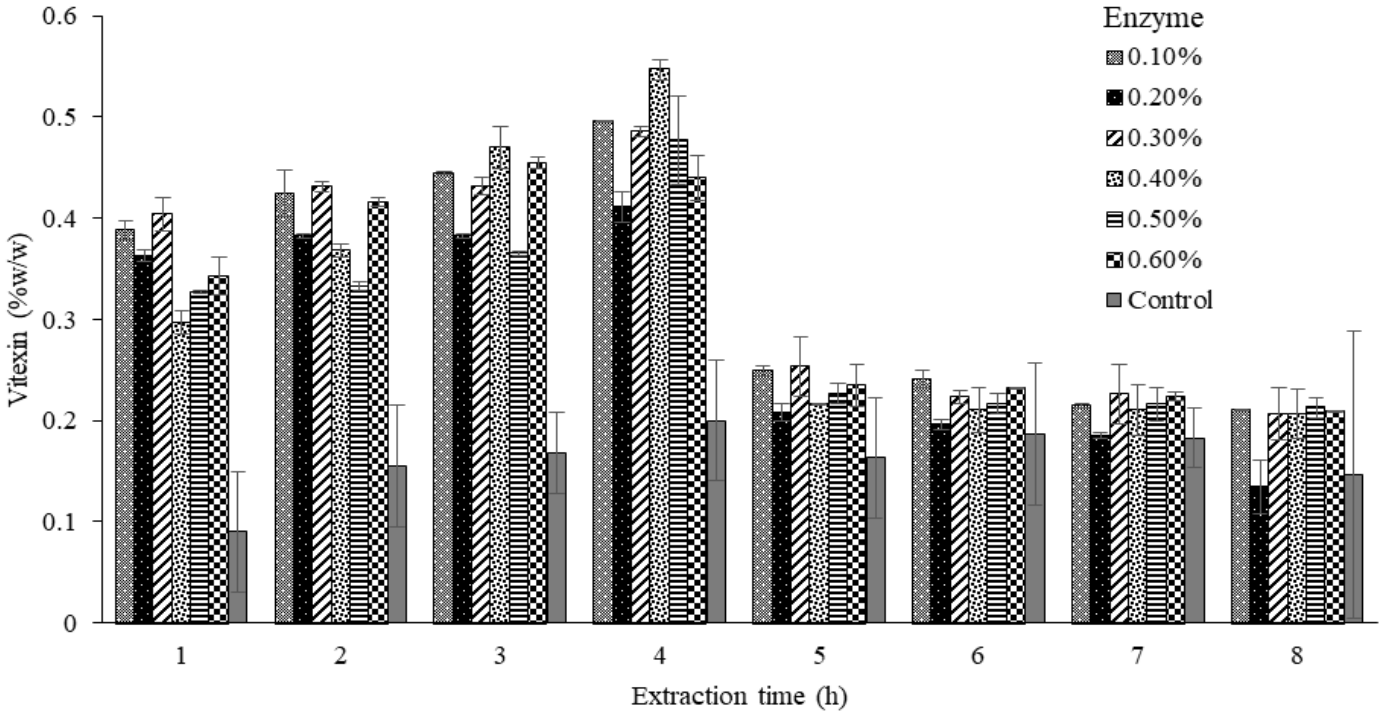

Figure 2. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:10 g/mL compared to control sample (50°C; pH 4.8; 8 h; n=6).

differed by 93% increase in the same ratio. The higher yield of vitexin compound from this method is due to the ability of hydrolytic enzyme to degrade or disrupt cell walls and membranes, thus enabling better release and more efficient extraction of bioactive compounds (Puri et al., 2012). It is also observed that at all concentrations applied the yield of vitexin drops for about 50 to 70% from 4 to 5th hour of extraction in this ratio. Parameter of time gives important effects in hydrolytic enzymes activity.

The longer the hydrolysis time, the more sufficient was the degeneration of Mas Cotek plant cells, and the more complete was the enzymatic hydrolysis. Thus, as the hydrolysis time increased, the quality of compounds will be decreased. This pattern is however consistent through all cellulase concentrations from 0.1 to 0.6% where from the 1st to 4th hour of extraction, the yield appeared to incline gradually but levelled off at the 5th hour, leaving a uniform trend until the end of THE experiment. Therefore, 4 h was taken as the best time of enzymatic mediated extraction in 1:10 (g/mL) sample-to-water ratio. Cellulase loading with 0.4% gave the best performance in this ratio. Compared to the lower concentrations, the increase in the cellulase dosage to 0.4% could be more helpful in the extraction of vitexin compounds in this ratio. However, as cellulase activity decreased during the hydrolysis process, the falloff pattern in the graph can be observed from the 4th to the end of extraction time. This deactivation is partially caused by the irreversible adsorption of cellulase on cellulose; in this study referred to as plant cell. Also, increasing the cellulase concentration to 0.6% did not contribute to the higher yield of vitexin in this ratio.

The second batch of EnAE was studied using 1:20 (g/mL) sample-to-water ratio as shown in Figure 3. In this ratio, cellulase concentration of 0.1% gave the highest yield of vitexin (0.530%) at the 4th hour of extraction.

A steep increase was observed in the pattern of yield from the 1st to 4th hour of extraction at all concentrations and then decreased gradually at the 5th hour until the 8th hour of extraction process. The same pattern was observed in the previous ratio as the recovery of active compounds from the plant sample influenced the extraction time and reflects the conflicting actions of solubilization and analyte degradation by oxidation with an increasing time. It was also found that increasing amounts of cellulase concentration to 0.6% lowered the yield of vitexin compared to other concentrations in this ratio. Other than the chances of phenolic compound oxidation at longer extraction time, at some point, the hydrolysis process is more adequate with the increasing of enzyme amount and caused limited amount of substrates. In average, the yield of vitexin in 1:20 (g/mL) ratio decreased by 20% compared to the average yield in 1:10 (g/mL) ratio.

The low yield of vitexin from EnAE in enzyme concentration of 0.6% at 7 and 8th hour of extraction

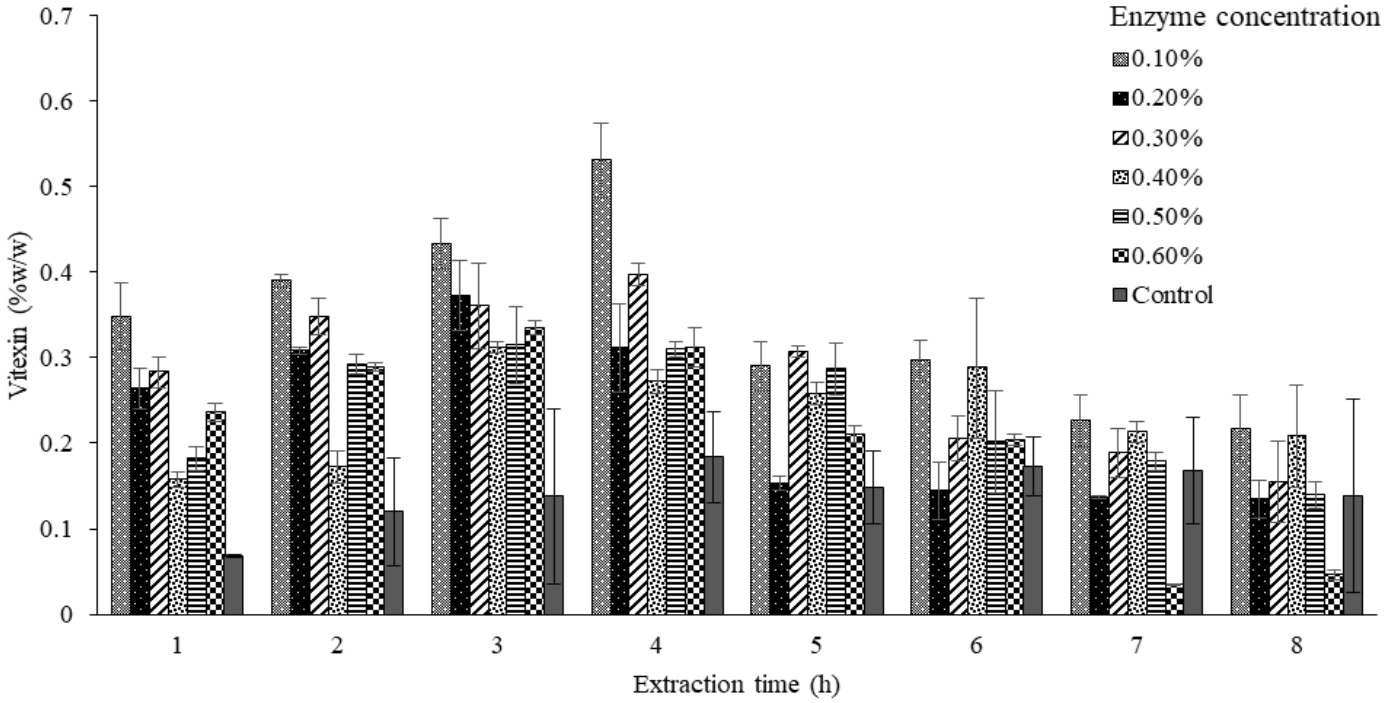

Figure 3. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:20 g/mL compare to control sample (50°C; pH 4.8; 8 h; n=6).

compared to the control sample were also observed in this ratio with 0.79 and 0.67-fold decreased, respectively. There are some factors that might affect the low yield from enzymatic-assisted extraction of vitexin compound. As more cellulosic cell wall digested by cellulase enzyme, more compounds will migrate into the surrounding medium. The released of unwanted and inactive compounds will cause the low detection of compound of interest (Patindol et al., 2007; Zhao et al., 2016).

The last batch of EnAE was carried out using 1:30 (g/mL) sample-to-water ratio as shown in Figure 4. The highest yield of vitexin (0.521%) was recorded from 0.2% cellulase concentration at the 3rd hour of extraction. This was however contrary to the findings obtained in the earlier two tested ratios. However, in respect of average yield, the value of vitexin from ratio 1:30 (g/mL) was lower than the value of vitexin from ratio 1:10 (g/mL) and the decrease was recorded by 11%. The percentage yield of vitexin dropped significantly with further increase in sample-to-water ratio, indicating that the reduced vitexin yield is likely to be resulted from the lowest solid content. As the highest average of vitexin obtained with the lowest ratio (1:10 g/mL) with the highest solid content, this ratio is the best choice and more acceptable due to lesser water requirement.

At the 7 and 8th hour of extraction, the yield of vitexin from 0.3 and 0.2% cellulase concentration observed were 67 and 11%, respectively. During the enzymatic-mediated extraction process, chemical reactions might happen on the complicated chemical composition in vitexin compound. Flavonoids (with phenolic hydroxyl group) and triterpenoids (with carboxyl group) present in the Mas Cotek leaves extracts may increase acidity of the extraction solution as it was already at pH 4.8. Under acidic condition, there may be a series of chemical reactions to cleave the glucosidic bonds in vitexin, resulting in different extraction values during the process at different sample-to-water ratio and cellulase concentrations (Bansal et al., 2009).

In terms of monetary aspect, increasing the dosage of cellulase enzyme in the process to a certain extent can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process (Sun and Cheng, 2002). Cellulase concentration of 0.4% was chosen as the best concentration in assisting the extraction of vitexin compound from Mas Cotek leaves sample. Different sample-to-water ratio gave different effects on the hydrolysis of plant cells at different cellulase concentrations. The differences might be due to the substrate (sample) as main factor, which caused the increase in yield and reaction rate at low substrate level and substrate inhibition at higher substrate concentration that leads to the low rate of hydrolysis.

When compared with the control sample (without cellulase-mediated), it was observed that even with small ranges of cellulase concentration, there are small differences in the optimum extraction time from both methods. The optimum time for control sample can be

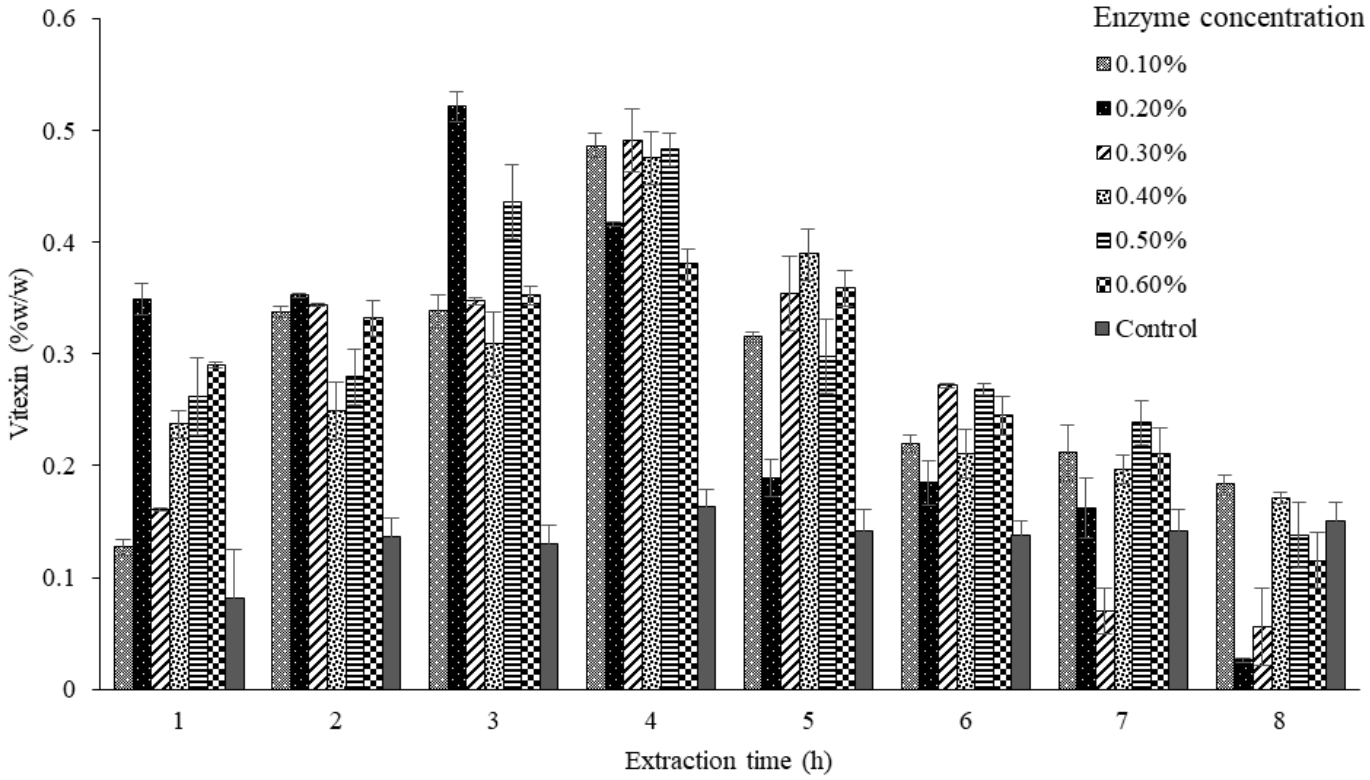

Figure 4. The effects of different cellulase concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6%) in the yield of vitexin compound from Mas Cotek leaves extract in sample-to-water ratio of 1:30 g/mL compare to control sample (50°C; pH 4.8; 8 h; n=6).

observed at the 4 and 5th hour of extraction while the 3th or 4th hour can be observed from EnAE. It showed that the use of enzyme significantly increased the rate of extraction by promoting the release of vitexin compound at shorter time ranges. The significance of using small ranges of cellulase concentration also can be justified with the unbalanced distribution of active compounds yield. This might be seen as a good finding because the used of more amount of enzyme will be more costly in terms of economic value.

The significant differences in the yield of vitexin achieved from enzymatic-assisted extraction using cellulase enzyme compared to yield obtained from control samples was the result of plant cellulose hydrolysis. Cellulose exists at nanometer scale in native plant cell walls as a microfibril network embedded in matrices of hemicelluloses, pectins and lignin (Liu et al., 2010). Cellulase is a class of enzyme that has the synergistic reaction to efficiently break down this chemically and physically complex plant cell wall polymeric network. This type of enzyme however plays different roles cooperatively in the hydrolysis of cellulose where some cleave to the cellulose chain from the middle into fragments containing 4 to 5 glucoses, some breakdown these fragments into smaller units of two glucoses, and some finally turn these small units into single glucose (Kumar et al., 2008). When more cellulose turns into simple glucose, more bioactive compound contained in the plant cell wall will be released into the surrounding medium without the aid of high temperature and energy. In summary, compared to control sample, cellulase-assisted extraction can break the cell wall under mild conditions to accelerate the dissolution of active compounds, which also reduce the extraction time, avoid the heat damage to compounds, improve the extraction yield of compounds and reduce the use of organic solvents and costs (Cao et al., 2014).

Glucose concentration liberated from sample

Another approach to indicate the efficiency of cellulase enzyme to cellulose degradation during the extraction is to calculate the amount of sugar released at every hour the experiment was conducted. Enzymatic hydrolysis provides a method to convert cellulose into glucose at high yields without reducing sugar product degradation. Cellulase enzyme used converted the hydrolysed cellulosic cell wall of Mas Cotek leaves into small particles of glucose. A good recovery of active compounds indicates that the Mas Cotek cell wall was more effectively degraded by the cellulase enzyme,

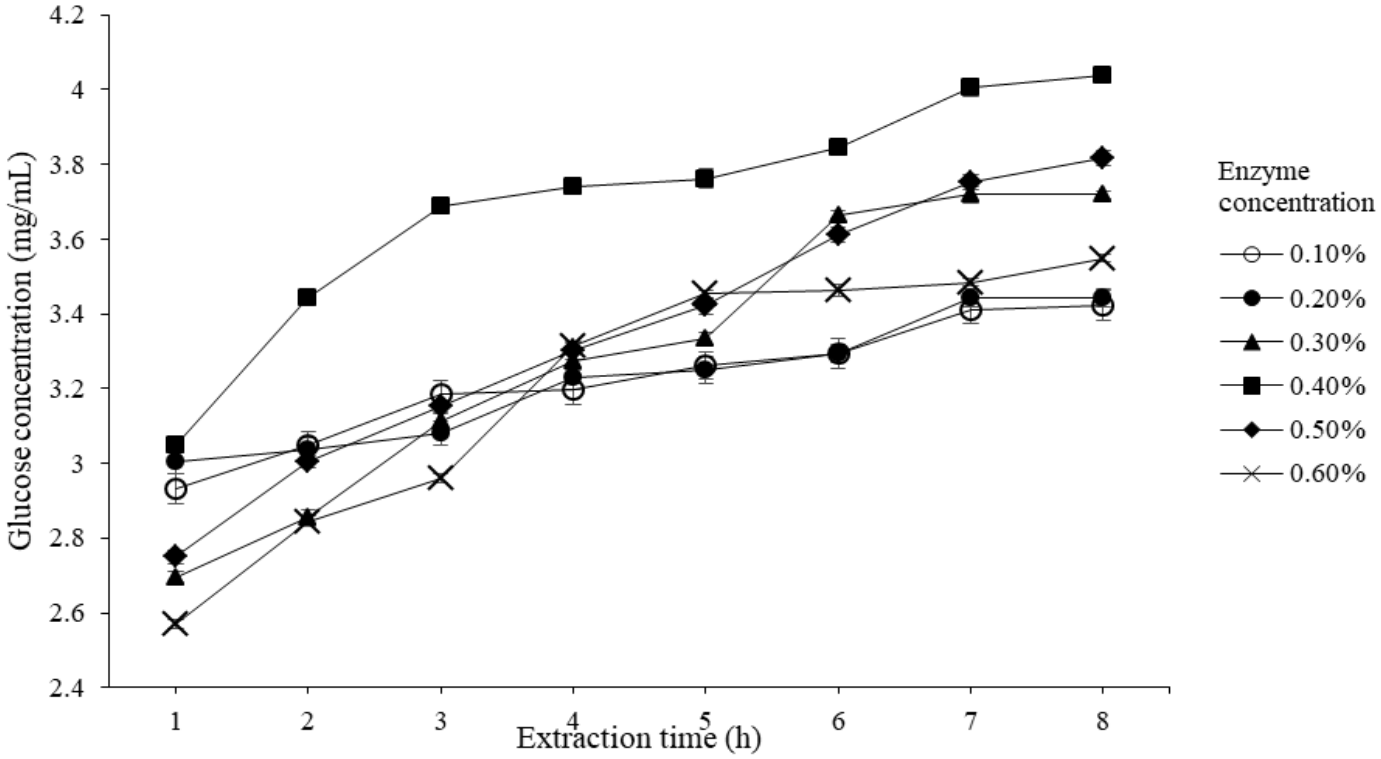

Figure 5. Glucose concentration determined in Mas Cotek leaves extract with different cellulase concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6% (50°C; pH 4.8; 8 h; n=6).

leading to the release of most of the compounds and other materials enmeshed within the cells into the aqueous medium. Therefore, the extracted solutions were sampled every hour to measure the concentration of glucose content in each sample. Figure 5 shows the glucose content in EnAE.

The reducing sugars released in all enzyme concentrations in this study were always determined by linear regression as glucose equivalents. The highest concentration of glucose was 4.039 mg/mL recorded from 0.4% cellulase concentration at the 8th hour extraction. The graph shows an increasing trend from 1st to 8th hour of extraction for all cellulase concentration. It was also observed that 0.4% cellulase concentration gave the highest glucose concentration compared to other concentration by 6%. The reducing sugars of the treated samples increase with cellulase concentration from 0.1 to 0.4%. However, a decrease is verified at higher cellulase concentration (0.5 and 0.6%) due to the removal of the broken chain by excess of enzyme and less accessibility of cellulose (Cavaco-Paulo and Almeida, 1994). Thus, any further increment in the cellulase concentration of more than 0.4% may not favour the release of reducing sugar into the extracts.

Other cellulase concentrations gave slight difference in glucose concentration throughout the extraction. The high glucose concentration in 0.4% cellulase concentration supported the findings in EnAE as more compounds were extracted in the same cellulase concentration compared to others. Enzymatic breakdown of cellulose to reducing sugars was done by enzymatic hydrolysis of the glucosidic bond. As more cellulose cell wall were digested by the enzyme, more active compounds escaped to the surrounding medium. Also, more production of glucose was determined in the sample. These findings were contributed by the mechanism of hydrolysis by cellulase enzyme complex. Beta-glucosidase hydrolyzes cellobiose, a disaccharide of beta-glucose and the product of cellulose hydrolysis by cellulase, into glucose (Sousa Jr. et al., 2011).

Different cellulase concentrations gave different amount of glucose production. Even though the range of concentrations used was small, there are a lot of factors that may contribute to these differences. In concentrations other than 0.4%, pectin hinders the hydrolysis of cellulose in the Mas Cotek leaves cell wall, and must be hydrolysed for cellulase to further hydrolyze other cell wall polysaccharides. Steric hindrance of cellulose hydrolysis by pectin is supported by the cell wall model for flowering plants proposed by Carpita and Gibeaut (1993), which stated that a pectin matrix surrounds cellulose fibers coated with xyloglucan, a hemicellulose. It was also found that the hydrolysis is thus a two-substrate reaction involving both cellulose and

water. While there has been considerable interest in the cellulose-enzyme interactions as well as on the cellulose composition, limited attention has been paid to the role of water in the process. During the enzymatic hydrolysis, cellulose-water interactions occurred. As the action of the enzyme system is to breakdown and loosen the cellulose, more water will be introduced into the structure and provided better access for the enzymes.

To date, there are no specific data on the use of dinitrosalycilic acid (DNS) colorimetric method to determine the amount of reducing sugars released as a result of hydrolysis of cellulase-mediated extraction of Mas Cotek leaves. In the present study, the obtained optimum values of glucose concentrations and the yield of active compounds was complementary to one another. In this process, cellulase enzyme (endoglucanse, exoglucanase and β-glucosidase) was used to convert cellulose into sugars. Due to the specific role of enzyme, for example while endoglucanase cleaves the internal bonds of cellulose, the exoglucanase and β-glucosidase give complete conversion of cellulose into sugars, a mixture of these enzymes is required (endoglucanases and exoglucanses for the hydrolysis of cellulose into cellobiose and β-glucosidases for the conversion of cellobiose to final product of glucose) (Bhaumik and Dhepe, 2005). It is useful to demonstrate the relative contribution of endoglucanase, exoglucanases and β-glucosidases during a time course hydrolysis of this cellulosic substrate (Silveira et al., 2014). Furthermore, this procedure gave a new insight into the synergy that exists among different components of the cellulase system.

Conclusion

In this study, cellulase enzyme at six different concentrations was tested to improve the release of vitexin yield from Mas Cotek leaves. The optimal conditions regarding the enzyme concentration and sample-to-water ratio were identified. The yield of vitexin compound was much higher in cellulase-mediated extracts of Mas Cotek leaves compared to control. This creates new opportunities for potential applications of this enzyme-assisted process. However, the long extraction time limits the economic potential. Further improvement might be required by coupling cellulase hydrolysis to other techniques such as ultrasonic extraction.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Assessment of genetic diversity among cassava landraces using single nucleotide polymorphic markers

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This study seeks to determine the genetic diversity among cassava landraces using single nucleotide polymorphic (SNP) markers. One hundred and five cassava landraces were assayed with 195 SNP markers. Major allele frequency varied from 0.500 to 0.942 with an average of 0.728. Average gene diversity, heterozygosity and polymorphic information content (PIC) were 0.359, 0.314 and 0.286 respectively. These values were generally high considering the bi-allelic nature of SNPs, hence the cassava landraces studied showed moderate to high genetic diversity. This suggests availability of unique and useful alleles that could be exploited for breeding purposes. Inclusion of these landraces in our crop improvement activities will enhance the development of farmer preferred cassava varieties. SNP markers used for the study were highly informative, polymorphic and revealed good estimates of genetic diversity among the landraces. Higher level of genetic variation was observed within population based on analysis of molecular variance (AMOVA). Principal component analysis (PCA) and cluster analysis also grouped landraces into three distinct clusters; however, they did not group in accordance to geographical origin. This could be due to high frequency of germplasm exchange between farmers and subsequent change of the name of the same cultivar. Results from this study may contribute significantly to cassava breeding and germplasm conservation programs.

Key words: Genetic diversity, single nucleotide polymorphisms (SNPs), polymorphic information content (PIC), polymorphic, alleles, heterozygosity, germplasm.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), member of the family Euphorbiaceae is a major root crop cultivated in most countries in sub-Saharan Africa. It is a staple for millions of households (Rabbi et al., 2014) and a subsistence crop due to its flexibility in planting and harvesting times. Cassava serves as a food security crop because of its high source of carbohydrate, ability to thrive under different climatic conditions (Tumuhimbise et al., 2014) and multipurpose uses for human consumption, animal feed and industrial applications (Rabbi et al., 2012). In Ghana, cassava is a major root crop and it is cultivated in all regions in the country. The livelihoods of about 70% of smallholder farmers depend on the crop. Over the years, a lot of improved cassava varieties

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have been developed and disseminated in Ghana. However, farmers still keep diverse sets of landraces on their farms, although they may be low yielding and susceptible to some biotic and abiotic stresses. These landraces may have increased genetic diversity which could promote gene flow through hybridization (Turyagyenda et al., 2012). Thus, landraces are important genetic resources (Mtunguja et al., 2014) for breeding and other crop improvement activities. They have different attributes which may present favorable characteristics that can contribute to food and nutritional security since it gives species the ability to adapt to changing environments including new pests, new diseases and new climatic conditions. They also provide opportunity for plant breeders to develop new and improved cultivars with desirable characteristics, for *in situ* conservation and studies on genetic diversity and evolution. To guarantee the security of these genetic resources, landraces must be maintained to avoid losses. However, they need to be characterized before they are maintained or conserved. This is necessary since there could be a mix up in the landraces where the same accession may have different names or different accessions may have the same name in different places. In addition, continuous exchange of planting materials between different farmers through both formal and informal distribution systems, make pedigree information limited and unreliable. Therefore, characterization of landraces is necessary as it will facilitate the removal of duplicates and creation of core collection for utilization by plant breeders. A prerequisite for any breeding programme is knowledge of the extent of genetic variability among cultivars. Such information guides the breeder to select distant parents to broaden the genetic base and to produce superior progenies.

Recent advances in molecular techniques have provided useful tools for characterization. Thus, molecular markers are widely used in plant genetic research and breeding. These markers permit the detection of genetic differences among closely related landraces, highly polymorphic, more stable and less influenced by the environment (Tiago et al., 2016). Several molecular markers have been used to characterize cassava germplasm to identify genetic variability. These include Random Amplified Polymorphic DNA (Rimoldi et al., 2010), Amplified Fragment Length Polymorphism (Benesi et al., 2010), microsatellites (Gonçalves et al., 2017) and more recently, Single nucleotide polymorphic (SNP) markers (Mtunguja et al., 2017).

With recent advances in high-throughput genotyping technologies, SNPs are increasingly becoming markers of choice for plant genetic studies and breeding. This is because of its cost effectiveness, locus specificity and co-dominant nature (Ren et al., 2013). Expressed sequence tags (ESTs) are fragments of mRNA sequences derived through single sequencing reactions performed on randomly selected clones from cDNA libraries (Parkinson and Blaxter, 2009). EST collections have been used to detect SNPs in crops such as maize (Ching et al., 2002) and soybean (Zhu et al., 2003). These SNPs have proved to be effective in the characterization of these crops. Similar studies in cassava has also led to the detection of SNPs from ESTs (Lopez et al., 2005; Ferguson et al., 2012), of which 1,190 have been validated and could be used in characterizing cassava. The aim of this study was to assess the genetic diversity among cassava landraces in Ghana using SNP markers. Understanding the extent of genetic diversity among cassava landraces may enhance efficient utilization by breeders for improving the crop.

MATERIALS AND METHODS

Source of germplasm

A total of 105 cassava landraces were collected from farmers' field in four major cassava growing zones (the rain forest, deciduous forest, transition and coastal savannah zones) of Ghana.

Screen house establishment

The planting materials were sprouted at the experimental station of the CSIR-Crops Research Institute, Kumasi, Ghana (6°41'N, 1°28'W).

Molecular characterization

DNA extraction

Total genomic DNA was extracted using standard procedures according to Egnin et al. (1998). About 200 mg of freshly harvested apical leaves of each accession were ground in liquid nitrogen into fine powder. Eight hundred microliters of extraction buffer (50 mM Tris HCL, pH 8.0, 300 mM NaCl, 20 mM EDTA, 20% PVP, 1.5% Sarcosine and 0.1 g/L $Na_2S_2O_2$) were used to lyse nuclear membranes.

Proteins and polysaccharides were precipitated by adding 400 µl of 5 M potassium acetate (instead of 800 µl of phenol chloroform isomyl alcohol) as used by Egnin et al. (1998) and the samples centrifuged at 13,000 rpm for 10 min. RNA was removed by adding 4 µl RNase A (10 mg/ml) and incubated at 37°C for thirty minutes. DNA was precipitated using 700 µl of ice-cold isopropanol and centrifuged at 13,000 rpm for ten minutes. Eighty percent ethanol was used to wash DNA and centrifuged at 13,000 rpm for five minutes. Ethanol was discarded and DNA pellets were air-dried at room temperature. DNA pellets were resuspended in 200 µl 1X TE (Tris-ethylenediaminetetracetic acid) buffer after which quality of DNA was determined on 0.8% (w/v) ethidium bromide stained agarose gel. The purity and quantification of DNA was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) with a spectrophotometer (Biochrom Libra S12).

SNP genotyping

KASPar technology was employed for SNP genotyping at the KBiosciences laboratories (United Kingdom). One hundred and ninety-five SNP markers developed from the Generation Challenge Programme (GCP) were used for the genotyping.

Table 1. Constituent reagent volumes for KASP genotyping mix.

Component	Volume (µl)
DNA	5.00
Mastermix	5.00
Primermix	0.14
Total reaction volume	10.00

Table 2. The KASP thermal cycling program.

Temperature/Time	Number of cycles
94°C for 20 s	Hot-start activation
94°C for 20 s 61-55°C for 60 s (dropping 0.6°C per cycle)	10 cycles
94°C for 20 s 55°C for 60 s	26 cycles

The genotyping assay for KASPar consisted of two reagent components (KASP Primer mix and KASP Master mix) plus the DNA sample. The KASP Primer mix was made up of two allele-specific forward primers and one common reverse primer. The KASP Master mix contained the FAM and HEX specific FRET cassette system, Taq polymerase, dNTP's, 5-carboxy-X-rhodamine, succinimidyl ester (ROX) and $MgCl_2$ in an optimized buffer solution. The KASP Primer mix was combined with the KASP Master mix and added to the DNA samples (5 - 50 ng) to be genotyped (Table 1) in a 96-well plate and sealed using the Kube^{TM} heat-based sealer. The reaction was carried out in a standard thermal cycler with conditions comprising two temperatures (Table 2). After completion of the PCR run, the scan results were read, and allele call was generated from the KlusterCaller software. SNP markers that were monomorphic were considered non informative and were removed from further analysis. A total of 187 SNP markers were therefore retained for genetic diversity analysis.

Data analysis

The genetic analysis package PowerMarker version 3.2 (Liu and Muse, 2005) was used to generate the following genetic diversity parameters: gene diversity, heterozygosity and polymorphic information content (PIC) (Bostein and White, 1980). PIC values were calculated with the equation: $PIC=1-\Sigma P^2_i-\Sigma\ 2P^2_i\ P^2_j$ Where: ΣP^2_i = sum of each squared i^{th} haplotype frequency. The software was used to calculate genetic distances among genotypes using the Euclidean method and neighbour-joining (NJ) algorithm (Nei, 1973) to construct a dendrogram from the distance matrix using MEGA 5.2 software (Tamura et al*.,* 2007) embedded in PowerMarker. Analysis of molecular variance (AMOVA) and principal component analysis were performed using GenAlEx 6.4 software (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

Genetic diversity

Genetic variation among genotypes is important for sustainable use of genetic resources to meet the demand for future food security as well as conservation strategies. This study was conducted to establish the genetic diversity among cassava landraces. Polymorphism frequencies are an important criterion that can be used to assess the value of molecular markers for germplasm characterization (Singh et al., 2013). From a total of 195 SNPs markers used for the study, 187 (96%) were polymorphic and 8 (4%) were monomorphic. The polymorphism observed in this study can be attributed to the fact that cassava landraces used for the study were diverse. In this regard 187 out of 195 SNPs used provided adequate informative polymorphism to evaluate genetic diversity of the cassava landraces. The high number of polymorphic SNPs is consistent with the mode of reproduction, genetic breeding system, and level of genetic variation in cassava. The results of this study were in close agreement with findings by Oliveira et al. (2014) who reported 1.5% of monomorphic SNPs when 1,280 cassava accessions were analyzed with 402 SNP markers.

PIC is a measure of the informativeness of a marker. The higher the PIC value the more informative the marker. PIC values varied from 0.049 to 0.375 with an average of 0.286 (Table 3), and approximately 56% of SNPs had estimates over 0.30. This suggests that the SNPs were informative and could discriminate among genotypes, hence could be candidate markers for genetic variability studies. Although the SNPs were informative, PIC values obtained with SNPs are generally lower compared to other molecular markers such as Simple Sequence Repeat (SSR) markers. In a recent study, PIC value varied between 0.030 to 0.780 when 89 cassava accessions were assayed with 35 SSRs (Adjebeng-Danquah et al., 2020). The low PIC value observed in this study is due to the bi-allelic nature of SNPs, hence

Table 3. Summary statistics of 105 cassava landraces assayed with 187 SNP markers.

Marker	Major allele frequency	Gene diversity	Heterozygosity	PIC
327-SNP	0.9	0.18	0.2	0.164
379-SNP	0.665	0.446	0.479	0.346
958-SNP	0.847	0.259	0.2	0.225
1920-SNP	0.512	0.5	0.476	0.375
2216-SNP	0.894	0.19	0.17	0.172
2257-SNP	0.756	0.369	0.274	0.301
2300-SNP	0.542	0.496	0.516	0.373
2496-SNP	0.973	0.052	0.053	0.05
6331-SNP	0.831	0.28	0.267	0.241
6453-SNP	0.709	0.413	0.363	0.328
6464-SNP	0.954	0.088	0.051	0.084
6630-SNP	0.869	0.227	0.216	0.201
6780-SNP	0.594	0.482	0.322	0.366
6889-SNP	0.556	0.494	0.444	0.372
6912-SNP	0.882	0.208	0.236	0.186
6922-SNP	0.95	0.095	0.056	0.09
7138-SNP	0.833	0.278	0.313	0.239
7239-SNP	0.807	0.311	0.26	0.263
7259-SNP	0.925	0.139	0.129	0.13
7434-SNP	0.825	0.289	0.35	0.247
Me_v4_MEF_c_3242	0.506	0.5	0.354	0.375
Me_v4_MEF_c_1018	0.511	0.5	0.348	0.375
Me_v4_MEF_c_1175	0.787	0.335	0.319	0.279
Me_v4_MEF_c_1183	0.527	0.499	0.598	0.374
Me_v4_MEF_c_1220	0.876	0.217	0.135	0.193
Me_v4_MEF_c_1246	0.669	0.443	0.413	0.345
Me_v4_MEF_c_1278	0.702	0.418	0.333	0.331
Me_v4_MEF_c_1320	0.75	0.375	0.364	0.305
Me_v4_MEF_c_2980	0.579	0.487	0.305	0.369
Me_v4_MEF_c_2990	0.604	0.478	0.659	0.364
Me_v4_MEF_c_2337	0.648	0.456	0.432	0.352
Me_v4_MEF_c_2363	0.546	0.496	0.517	0.373
Me_v4_MEF_c_2366	0.523	0.499	0.609	0.374
Me_v4_MEF_c_2851	0.689	0.428	0.432	0.337
Me_v4_MEF_c_2873	0.75	0.375	0.221	0.305
Me_v4_MEF_c_1387	0.882	0.208	0.191	0.186
Me_v4_MEF_c_2286	0.601	0.48	0.606	0.365
Me_v4_MEF_c_2283	0.733	0.392	0.395	0.315
Me_v4_MEF_c_3057	0.963	0.071	0.074	0.069
Me_v4_MEF_c_1447	0.506	0.5	0.41	0.375
Me_v4_MEF_c_1527	0.516	0.499	0.293	0.375
Me_v4_MEF_c_1566	0.705	0.416	0.35	0.329
Me_v4_MEF_c_1617	0.795	0.326	0.289	0.273
Me_v4_MEF_c_1637	0.876	0.217	0.135	0.193
Me_v4_MEF_c_1645	0.81	0.307	0.287	0.26
Me_v4_MEF_c_1679	0.689	0.429	0.444	0.337
Me_v4_MEF_c_1892	0.974	0.05	0.031	0.049
Me_v4_MEF_c_1919	0.849	0.256	0.215	0.223
Me_v4_MEF_c_1940	0.565	0.492	0.588	0.371
Me_v4_MEF_c_1945	0.747	0.378	0.11	0.306

Table 3. Contd.

Me_v4_MEF_c_1947	0.833	0.278	0.204	0.239
Me_v4_MEF_c_1958	0.864	0.236	0.152	0.208
Me_v4_MEF_c_1977	0.705	0.416	0.295	0.33
Me_v4_MEF_c_2034	0.545	0.496	0.455	0.373
Me_v4_MEF_c_2043	0.89	0.196	0.198	0.176
Me_v4_MEF_c_2051	0.736	0.388	0.484	0.313
Me_v4_MEF_c_2120	0.88	0.211	0.109	0.188
Me_v4_MEF_c_2124	0.759	0.366	0.235	0.299
Me_v4_MEF_c_2189	0.53	0.498	0.28	0.374
Me_v4_MEF_c_2195	0.776	0.348	0.269	0.287
Me_v4_MEF_c_2226	0.823	0.292	0.312	0.249
Me_v4_MEF_c_2236	0.724	0.4	0.368	0.32
Me_v4_MEF_c_2384	0.714	0.408	0.327	0.325
Me_v4_MEF_c_2402	0.672	0.441	0.355	0.344
Me_v4_MEF_c_2409	0.565	0.491	0.848	0.371
Me_v4_MEF_c_2419	0.569	0.491	0.238	0.37
Me_v4_MEF_c_2437	0.823	0.291	0.293	0.249
Me_v4_MEF_c_2447	0.778	0.345	0.239	0.285
Me_v4_MEF_c_2448	0.523	0.499	0.465	0.374
Me_v4_MEF_c_2456	0.741	0.384	0.42	0.31
Me_v4_MEF_c_2486	0.827	0.287	0.327	0.246
Me_v4_MEF_c_2510	0.841	0.268	0.273	0.232
Me_v4_MEF_c_2524	0.753	0.372	0.385	0.303
Me_v4_MEF_c_2552	0.683	0.433	0.366	0.339
Me_v4_MEF_c_2562	0.5	0.5	0.407	0.375
Me_v4_MEF_c_2653	0.606	0.477	0.511	0.363
Me_v4_MEF_c_2726	0.601	0.48	0.245	0.365
Me_v4_MEF_c_2748	0.939	0.114	0.101	0.107
Me_v4_MEF_c_2758	0.545	0.496	0.477	0.373
Me_v4_MEF_c_2801	0.608	0.477	0.462	0.363
Me_v4_MEF_c_2888	0.86	0.241	0.191	0.212
Me_v4_MEF_c_2905	0.947	0.1	0	0.095
Me_v4_MEF_c_2909	0.571	0.49	0.19	0.37
Me_v4_MEF_c_3070	0.563	0.492	0.292	0.371
Me_v4_MEF_c_3081	0.734	0.39	0.323	0.314
Me_v4_MEF_c_3094	0.771	0.353	0.394	0.291
Me_v4_MEF_c_3120	0.634	0.464	0.588	0.356
Me_v4_MEF_c_3131	0.8	0.32	0.275	0.269
Me_v4_MEF_c_3137	0.812	0.306	0.325	0.259
Me_v4_MEF_c_3142	0.847	0.259	0.235	0.226
Me_v4_MEF_c_3155	0.727	0.397	0.381	0.318
Me_v4_MEF_c_3195	0.859	0.243	0.152	0.213
Me_v4_MEF_c_3197	0.554	0.494	0.096	0.372
Me_v4_MEF_c_3310	0.672	0.441	0.323	0.344
Me_v4_MEF_c_3336	0.89	0.196	0.176	0.176
Me_v4_MEF_c_3338	0.72	0.404	0.244	0.322
Me_v4_MEF_c_3343	0.606	0.478	0.465	0.363
Me_v4_MEF_c_3356	0.556	0.494	0.238	0.372
Me_v4_MEF_c_3361	0.824	0.289	0.245	0.248
Me_v4_MEF_c_3376	0.809	0.309	0.337	0.261
Minimum	0.5	0.05	0	0.049
Maximum	0.94	0.5	0.848	0.375

Table 3. Contd.

Mean	0.73	0.359	0.314	0.286
STD[a]	0.132	0.116	0.135	0.077
95% CI	0.021	0.019	0.022	0.012

PIC: Polymorphic information content; STD[a]: Standard deviation with 95% confidence interval.

PIC values can range from 0.000 to 0.500, compared with SSRs which are multiallelic and can have PIC values above 0.500 and up to 1.000. Consequently, results from this present study demonstrate that the set of SNPs used were sufficiently informative and can be used for cassava genotyping to establish the relatedness between genotypes. PIC values however, observed in this present study was higher than that observed (PIC=0.170) by Karim et al. (2020) where cassava collections were analyzed with 5600 SNPs. The discrepancy could be attributed to differences in composition of experimental material, number and selection of SNP markers used for the studies.

Major allele frequency (MaF) for all the markers was generally high. It ranged from 0.500 to 0.942 with an average of 0.728 (Table 3). More than 50% of the polymorphic loci showed a major allele frequency higher than 0.700 and 9 loci showed more than 0.900. This is an indication that all the markers were polymorphic.

Heterozygosity, which is a measure of allelic diversity at a locus varied from 0.000 to 0.848 with an average of 0.314 (Table 3). Approximately 91% of the estimates were lower than 0.500. In contrast gene diversity (GD) varied from 0.50 to 0.500 with an average of 0.359, and only 29% of GD estimates were greater than 0.450. As heterozygosity is a measure of genetic variation among genotypes, the average heterozygosity observed could be expected to correlate with moderate to high genetic diversity. Average heterozygosity values however, observed in this present study was lower than that observed by Ferguson et al. (2019), who used 1,536 SNPs to genotype 522 cassava accessions (0.366). This is expected since most of the accessions used were collected from South America which encompasses the presumed centre of domestication and diversity of the crop. Average gene diversity value observed in this present study was higher than that observed by Kamanda et al. (2020), where 5,634 SNPs were used to assay 183 provitamin-A cassava accessions (GD=0.190). The differences could be attributed to the experimental materials and selection of SNP markers used. The average gene diversity was however higher than the heterozygosity in this study, suggesting inbreeding or heterozygote deficit in relation to that expected under the Hardy Weinberg equilibrium in the case of natural populations. A similar trend (GD=0.300; heterozygosity=0.230) was also observed by de Albuquerque et al. (2018). The high allelic richness coupled with estimates of gene diversity suggests a moderate to high genetic diversity among the cassava landraces. With SNPs, diversity values are generally low, and this could be explained by the bi-allelic nature, hence, the maximum gene diversity for a SNP marker is 0.5. In addition, cassava is allogamous and there is the possibility of identifying several alleles per locus.

According to several reports, to get the same level of information as SSRs for genetic diversity studies, a larger number of SNPs (7 to 11 times or more as compared to SSRs) must be used (Filippi et al., 2015). It is therefore important that the number and selection of SNPs, as well as the use of a large panel of accessions is considered to prevent bias and enhance the accuracy of diversity studies (Emmanuelli et al., 2013). However, Kawuki et al. (2009) have also reported that the number of SNPs required for a diversity studies is also dependent on the nature of the genetic resources. Hence, the more diverse the genetic resources, the fewer the markers required. In this study, 187 polymorphic SNPs were able to detect the genetic diversity in the cassava landraces which suggests that the number of SNPs used is within a suitable range. This suggests that the cassava landraces are diverse, and it supports the assertion by Kawuki et al. (2009). A similar trend was observed by Oliveira et al. (2014) who used 402 polymorphic SNPs to assess 1280 cassava accessions (Table 3).

Analysis of molecular variance (AMOVA)

AMOVA based on the SNP marker data suggests higher within group variation which accounted for 99% of the total variation compared with variation between groups which accounted for only 1% of the total variation (Table 4). The results suggest that there is a higher genetic diversity within population than as seen between populations. This result is similar to studies by Pedri et al. (2019) where analysis of molecular variance revealed a within group variation of 92%.

Principal coordinates analysis (PCoA)

The principal coordinates analysis generated is graphically presented in Figure 1. The coordinates were calculated for axes 2 and 3 with positive eigen values. The two axes accounted for 50.81% of the total variation with the second axis (PCoA2) accounting for 22.16% and third axis (PCoA3) accounting for 28.65%. The PCoA showed loose clustering and cassava landraces were

Table 4. AMOVA of cassava landraces populations.

Source of variation	df	Sum of squares	Mean square	Estimated variance	% variance
Between populations	1	42.331	42.331	0.295	1
Within population	103	3083.640	29.938	29.938	99
Total	105	3125.971	72.269	30.233	100

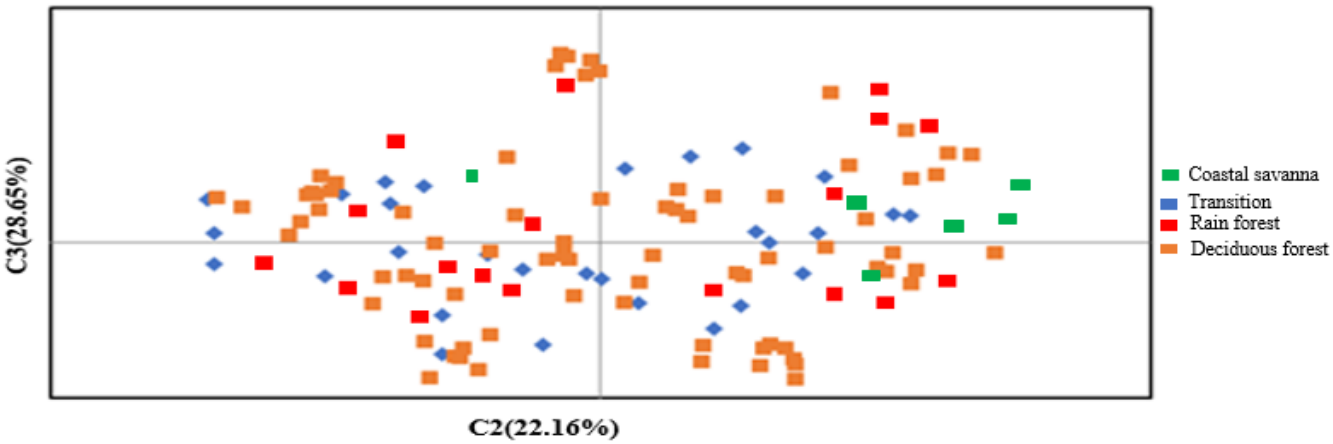

Figure 1. Principal component analysis generated by GenAlEx version 6.4.

dispersed in all four quadrants. However, those which clustered together could suggest that they share some common alleles. The PCA analyses, however, showed a similar result to the clustering supporting the presence of four populations**.**

Cluster analysis

The dendrogram was constructed using Nei's genetic distance (1973) and this separated the 105 cassava landraces into three main Clusters (I, II and III) and five distinct sub-clusters (Figure 2). Clustering did not reflect the zones from which these landraces were collected and subsequently no duplicates were found. Cluster I composed of landraces from the coastal savanna zone while all other landraces from the rain forest, deciduous forest and transition grouped across the other two Clusters (II and III).

Grouping of landraces from different agro-ecological zones across the different clusters could be attributed to the high frequency of germpalsm exchange among farmers. Within cluster II, three sub-clusters (A, B and C) were formed, while two sub-clusters (D and E) were formed in cluster III. Sub-cluster size varied from four to 65. The largest sub-clusters, C and D had 33% each of the cassava landraces, while sub-clusters A and E had 6% each and sub-cluster B had 22%. Cluster analysis with the SNP markers was useful in revealing three distinct groups which may have diverse morphological, agronomical, physiological and molecular characteristics that may provide valuable genetic resources. This may enable breeders select diverse parents for targeted crosses to develop superior progenies.

Conclusion

This study concluded that 187 out of 195 SNP markers were highly informative and polymorphic to distinguish among the cassava landraces. These SNPs could therefore be used for future genetic diversity studies in cassava. In addition, SNP markers were able to separate the landraces into different clusters. However, they were not grouped in accordance to their geographical origin. Furthermore, this study has confirmed that fewer SNPs could be used to assess the genetic diversity if the genetic resources are diverse. The cassava landraces used for this study showed moderate to high genetic diversity, suggesting the availability of useful and unique alleles for desirable traits that could be exploited for breeding purposes. Inclusion of such landraces in cassava improvement activities would enhance the development of end-user varieties that will be easily adopted by farmers and other stakeholders along the value chain. The information generated will contribute significantly to manage conserved germplasm, develop core collections from which parental lines could be selected to improve existing cultivars.

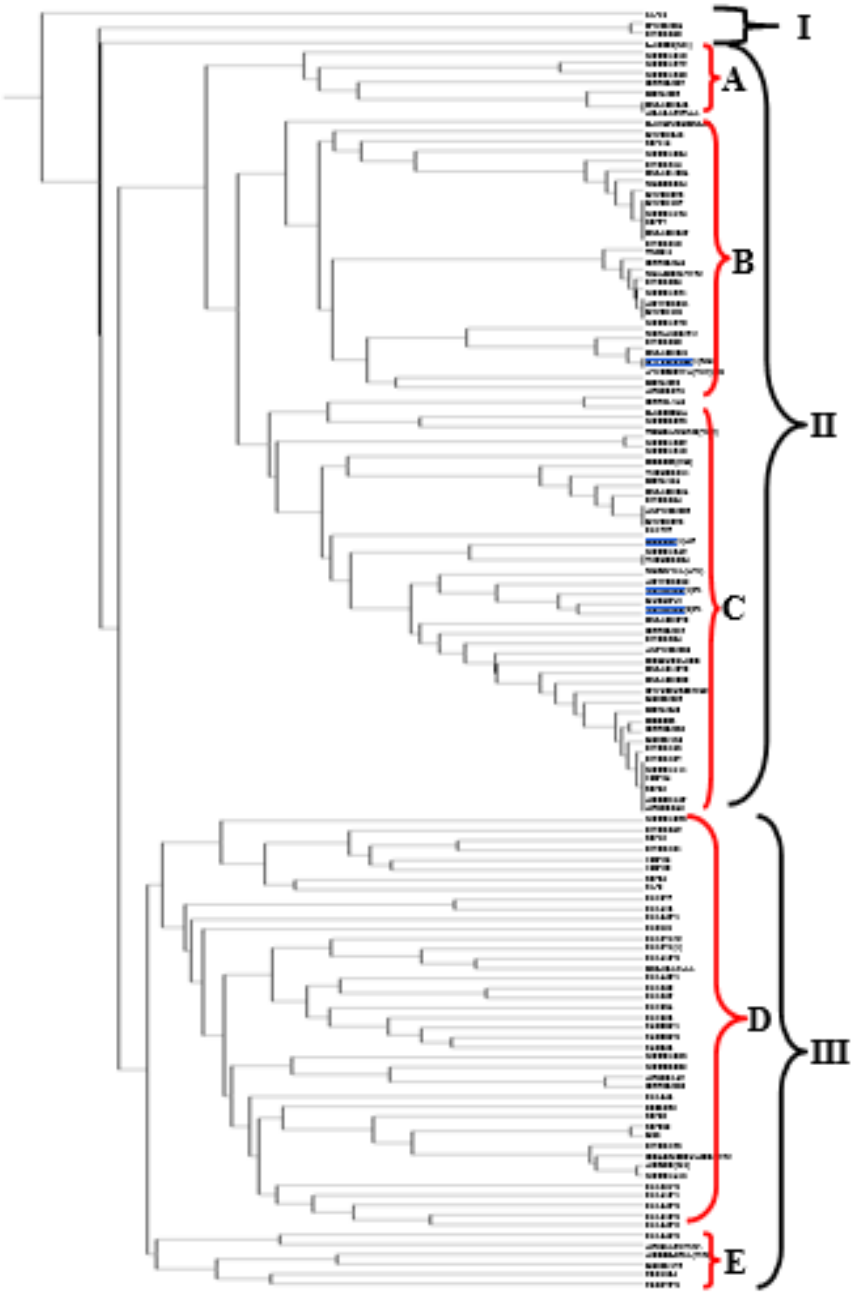

Figure 2. Dendrogram of 105 cassava landraces using 187 SNPs based on Nei's genetic distance (1973) generated by PowerMarker 3.0 software.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Cytotoxic effects of some essential oils on Mcf-7, Hfs and Hct116 cell lines

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This study investigates the chemical composition and cytotoxic effects of three essential oils of rosemary, lemon and orange, as well as its effects on breast cancer (MCF-7), human foreskin fibroblasts (HFS) and colorectal carcinoma (HCT116) using gas chromatography-mass spectrometry (GC-MS) methods, leading to identification of a different compound by GC-MS. Eicosapentaenoic acid (13.79%) and heptatriacotanol (13.79%) were the major constituents present in the orange oil. Cytotoxic activity was evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methods. Results revealed that essential oils significantly reduced the viability of MCF-7, HFS and HCT116 cells in a concentration-dependent manner. The evaluated essential oils could prove to be promising for future applications in the treatment of cancer-related diseases.

Key words: Anticancer activity, chemical composition, essential oil, cytotoxic effects, cell lines, Mcf-7, Hfs, Hct116, gas chromatography-mass spectrometry (GC–MS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

INTRODUCTION

Medicinal plants have been used in healthcare since time immemorial for well-being and bio-prospecting of new plant-derived drugs. Cancer is a group of disease that endangers human life and has currently become the first leading cause of death in the world. The normal cells control the production and release of growth factors, which regulate cell growth/proliferation, thus ensuring cellular homeostasis and maintenance of normal tissue architecture; however, in the case of cancer, cells increase proliferatively because these signals are deregulated and thus, homeostasis within the cell is disrupted (Hanahan and Weinberg, 2011).

Cancer cells can signal normal neighboring cells, resulting in mutations/alterations in signaling pathways. These alterations stimulate the release of growth factors which are supplied back to the cancer cells, enhancing their proliferation (Bhowmick et al., 2004; Cheng et al., 2008). Breast cancer is the most common form of cancer affecting women in the Kingdom of Saudi Arabia (KSA), and there is a steady increase in its patients (Amin et al., 2009).

Many pharmaceutical agents have been discovered by

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screening natural products from plants and are currently successfully employed in cancer treatment (Da Rocha et al., 2001). The exploration into natural products offers great opportunity to evaluate new chemical classes of anticancer agents. The plant-derived secondary metabolites products are expected to induce lesser side effects compared to synthetic drugs. Among plant derived compounds, essential oils (EOs) from aromatic plants have been reported to possess anticancer properties (Yu et al., 2011). EOs has also been reported to improve the quality of life of the cancer patients by lowering the level of their agony (Bhalla et al., 2013).

Citruses such as oranges and lemons provide multitude of health benefits resulting from their constituent compounds like bitter limonods, carotenoids, flavonoids, and folic acid which have been shown to prevent a variety of cancers and cardiovascular diseases (Mohamed et al., 2010).

Lemon is used traditionally to reduce high blood pressure, mental health, respiratory problems, arthritis, and treatment of diabetes, rheumatism and headaches. The essential oil of lemon showed fungi-toxicity against some fungi, anticancer and antibacterial potency (Al-Jabri and Hossain, 2018). The majority of chemical compounds in lemon fruits are alkaloids (Oikeh et al., 2016). Similarly, Omani sour lemon essential oil was composed of limonene (53.57%), a-terpineol (14.69%), b-pinene (8.23%), a-pinene (1.84%), b-myrcene (1.51%), a-terpinolene (4.33%), terpinen-4-ol (3.38%), cymene (1.80%), b-bisabolene (1.43%), b-linalool (0.85%) and E-citral (1.08%) (Al-Jabri and Hossain, 2014; Tadtong et al., 2015).

The plant *Rosmarinus officinalis* L., a member of the mint family Lamiaceae has many culinary and medicinal uses. The main polyphenols found in rosemary extract (RE) include the diterpenescarnosic acid (CA) and rosmarinic acid (RA) (Cuvelier et al., 1994), and also contains numerous phytochemicals such as flavonoids, glycosides, phytosterols and polyphenols; phytochemicals that are claimed to have antioxidant activity. The aim of this study is to analyze the chemical composition of three different essential oils (lemon, orange and rosemary), in addition to evaluating their cytotoxic activity against human carcinoma; breast cancer (MCF-7); human foreskin fibroblasts (HFS) and colorectal carcinoma (HCT116).

MATERIALS AND METHODS

Essential oils (EOs)

The following commercial EOs (lemon, orange and rosemary) were purchased from a GNC market (http://www.armalgnc.com/index.php?id_lang=1) in Makkah City, Saudia Arabia in October 2018, and the EOs were kept at 4°C in dark containers until they were used for the experiment. The oil samples were diluted in diethyl ether (1: 200) for GC-MS analysis.

Cell culture

The cell lines including, breast cancer (MCF-7), human foreskin fibroblasts (HFS) and colorectal carcinoma (HCT116) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Institutional ethical approval was obtained for the use of cancer cells in this study.

GC/MS analysis of essential oils

The oil samples were diluted in diethyl ether (1: 200) for GC-MS analysis using a capillary gas Chromatography (model Trace GC ULTRA, Thermo Scientific) directly coupled to ISQ Single Quadruple MS.

The GC/MS analysis was performed on a TG-5MS non-polar 5% phenyl methylpolysiloxane capillary column (30 m × 0.25 mm ID × 0.25 µm). The oven temperature was maintained at 40°C for 3 min and heated slowly to 280°C at 5°C/min. The flow rate of carrier gas (Helium) was 1 ml/min, the injected sample volume was 10 µl, split less injection technique, and ionization energy of 70 eV in the electronic ionization (EI) mode.

Identification of essential oils components

The compounds were identified by the retention index and the fragmentation pattern of the authentic compound mass spectra data (Chien et al., 2009). Few compounds were also identified by Wiley 9 and NIST08 commercial catalogue library (Database of National Institute Standard and Technology) having more than 62,000 patterns.

Cell growth

Cells were cultivated in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both GIBCO, Grand Island, NY, USA), 1% L-glutamine, 1% penicillin, and streptomycin, 10% serum, 100x, 1% streptomycin/penicillin/amphotericin B solution, and then periodically sub-cultured and transferred in a humid-CO_2 incubator (ShelLab, USA) at 5% CO_2, 37°C. Cell numbers were calculated using a Neubauer Chamber. The cells were thawed, allowed to grow, and subcultured until it reaches the number of cells needed which was 2,000,000 for all the plates for each type of cells.

This is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan. The cells (MCF-7, HFS and HCT116) were seeded with 200 µL DMEM/F12 medium containing bovine serum in 96-wells plate and incubated in 37°C for 24 h. Stock solutions of the three essential oils (Lemon oil, rosemary oil and orange oil) were prepared in DMSO (1%) and phosphate buffered saline (PBS) at different concentrations (50, 100, 150 and 200 µl/mL) for MTT assay.

After 72 h of cell incubation with different doses of the three essential oils (50, 100, 150 and 200 µl/mL), 20 µL MTT solution (5 mg/mL) was added to each well. After 4 h incubation at 37°C, the formazan was dissolved in DMSO. Finally, the optical density (OD) of wells was measured on a micro plate ELISA reader at 540 nm.

Normal human fibroblasts (wst-1) were used as control. In the first day, each well was seeded with 10,000 cells; and the next day, the media was replaced with drugs concentration. After 24 h, we apply with WST-1 kit for cell viability assay, 10 µl was added on each wells, incubated for 2 h, then read with the ELISA READER MACHIN at 540 nm. Similar approach was followed in the 48 h and 72 h incubation (Thani et al., 2014).

Each oil concentration was repeated eight times, standard curves for each sample (absorbance value against cell number) were

Table 1. GC/MS chemical profile of lemon oil.

No.	[a]R_t	Compounds name	Area (%)[b]	Molecular formula
1	7.41	D-Limonene	24.58	$C_{10}H_{16}$
2	19.61	Z,Z 3,15 Octadecadien-1-ol acetate	1.64	$C_{20}H_{36}O_2$
3	21.08	4-tert-Butylcyclohexyl Acetate	3.41	$C_{12}H_{22}O_2$
4	30.39	Diethyl Phthalate	60.61	$C_{12}H_{14}O_4$
5	30.70	1,2- Benzenedicarboxylic acid, diethyl ester	0.85	$C_{12}H_{14}O_4$
6	31.72	2,4- Imidazolidinedione,1-[[(5nitro2furanyl) methylene]amino]	0.76	$C_8H_6N_4O_5$
7	58.69	Á-d-Mannofuranoside, Ogeranyl-Squalene	4.86	$C_{16}H_{28}O_6$
8	59.61	6,9,12 Octadecatrienoic acid, methylester	0.87	$C_{19}H_{32}O_2$
9	59.60	Cis-5,8,11,14,17-Eicosapentaenoicacid	0.87	$C_{20}H_{30}O_2$

[a]Rt: retention time (min). [b]The percentage composition was computed from the gas chromatography peak areas.

Table 2. GC/MS chemical profile of orange oil.

No.	[a]Rt	Compounds name	Area (%)[b]	Molecular formula
1	3.07	Butanoic acid,2methyl,ethyl ester	16.54	$C_7H_{14}O_2$
2	12.02	Camphor	10.65	$C_{10}H_{16}O$
3	13.25	Cyclohexanol,5-methyl-2-(1methylethyl)	28.84	$C_{10}H_{20}O$
4	23.25	2(1Cyclohexenyl)Ethylamine	4.75	$C_8H_{15}N$
5	23.46	2,4 Undecadienal	6.39	$C_{11}H_{18}O$
6	24.67	Docosahexaenoicacid,1,2,3 propanetriylester	5.01	$C_{69}H_{98}O_6$
7	57.15	Ethanol, 2(9,12octadecadienyloxy)	4.85	$C_{20}H_{38}O_2$
8	57.15	Trilinolein	4.85	$C_{57}H_{98}O_6$
9	57.23	9 Octadecenoic acid, 1,2,3 propanetriylester,	4.59	$C_{57}H_{104}O_6$
10	57.32	cis13 Eicosenoic acid	4.68	$C_{20}H_{38}O_2$
11	58.72	1-Heptatriacotanol	13.79	$C_{37}H_{76}O$

[a]Rt: retention time (min). [b]The percentage composition was computed from the gas chromatography peak areas.

plotted, and cell viability was calculated based on the standard curves. Cell survival in the negative control was assumed to be 100% and IC_{50} was calculated using analysis of regression. Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls test was used to identify the differences among the groups. Significance was assumed at 5% level.

RESULTS

Essential oils by GC-MS

The analytical results of lemon, orange and rosemary essential oils are shown in Tables 1 to 3 respectively. The main components of the lemon oil were diethyl phthalate (60.61%) and D-Limonene (4.86%) followed by ۔-d-Mannofuranoside, Ogeranyl-Squalene (4.86%) as shown in (Table 1). Monoterpenes derived from citrus have been shown to inhibit human cancer cell proliferation and tumor growth through various mechanisms such as promoting cell apoptosis, inhibition of the expression of growth factors and cell cycle arrest (Chidambara Murthy et al., 2012). For the orange oil, the Cyclohexanol, 5-methyl-2-(1-methylethyl) was detected at a level of (28.84%) as a major compound; followed by Butanoic acid, 2-methyl-, ethyl ester (16.54%), 1-Heptatriacontanol (13.79%) and Camphor (10.65%). On the other hand, Squalene (27.34%) was the major percentage of the rosemary oil followed by trans-Geranylgeraniol (24.82%) and Lucenin-2 (7.10%) as shown in Table 3.

Cell viability of essential oils

The cell viability was examined at a different concentration (50, 100, 150 and 200 µl/ml) of essential oils against 3 cancer cells (MCF-7, HFS and HCT116), and the results showed that the more sensitive cancer cell for the used essential oils was MCF-7 (breast cancer) cell line, followed by HFS (human foreskin fibroblasts) and HCT116 (colorectal carcinoma) with comparison to normal human fibroblasts using MTT assay after 24 h (Figure 1). Essential oils significantly reduced the viability of MCF-7, HFS and HCT116 cells in a concentration-dependent manner.

Table 3. GC/MS chemical profile of Rosmery oil.

No.	[a]Rt	Compounds name	Area (%)[b]	Molecular formula
1	34.1	Pestalone	5.60	$C_{21}H_{20}Cl_2O_6$
2	4.11	7-Bromo3[(4bromo3,3dimethylcyc lohexylidene) methyl]octahydro4,7adimethyl1Hindene2,4diol	5.37	$C_{20}H_{32}Br_2O_2$
3	50.64	9 Octadecenoicacid	7.85	$C_{18}H_{34}O_2$
4	50.80	àDMannopyranoside,methyl, cyclic 2,3:4,6bis(butylboronate) (CAS)	5.28	$C_{15}H_{28}B_2O_6$
5	58.72	Squalene	27.34	$C_{30}H_{50}$
6	58.76	TransGeranylgeraniol	24.82	$C_{20}H_{34}O$
7	59.47	9,12,15Octadecatrienoicacid,2,3bis[(trimethylsilyl)oxy]propyl ester,	7.10	$C_{27}H_{52}O_4Si_2$
	59.47	Lucenin 2	7.10	$C_{27}H_{30}O_{16}$
8	59.96	Betamethasone valerate	5.93	$C_{27}H_{37}FO_6$
9	60.13	Ethanol, 2(9,12 octadecadienyloxy)	5.17	$C_{20}H_{38}O_2$
10	60.29	Hexadecadienoic acid, methyl ester	5.53	$C_{17}H_{30}O_2$

[a]Rt: retention time (min). [b]The percentage composition was computed from the gas chromatography peak areas.

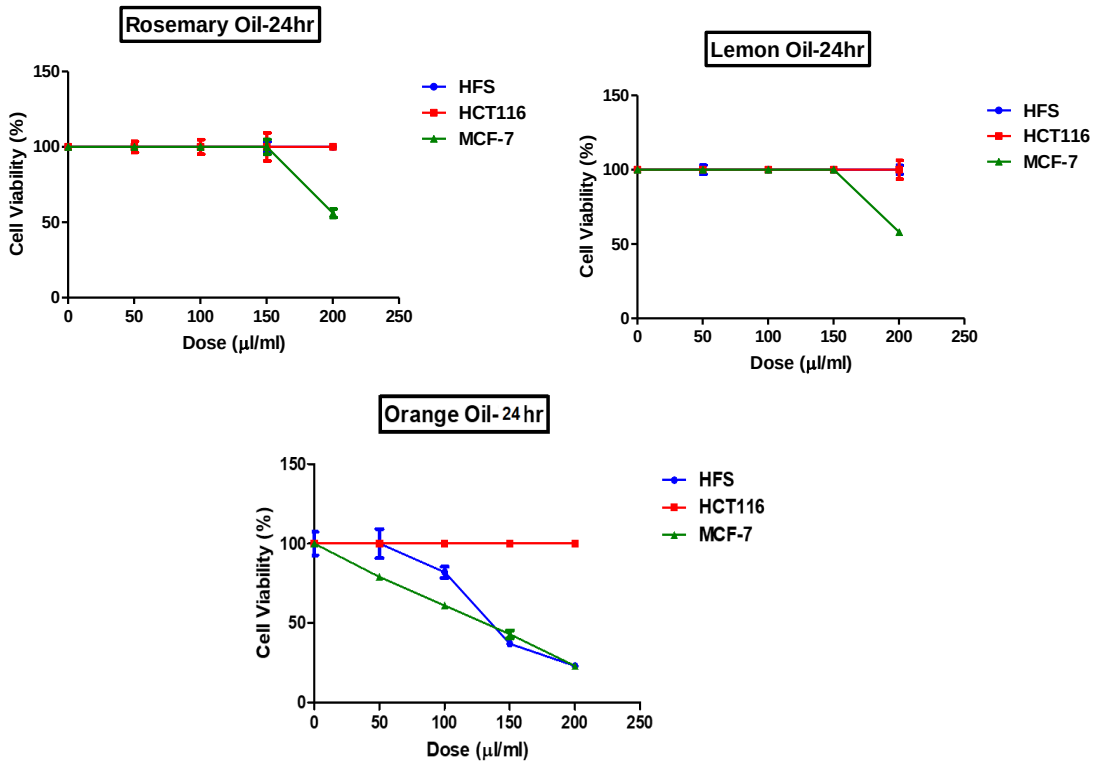

Figure 1. Percentage of cell viability on different concentration of essential oils against MCF-7, HFS and HCT116 using MTT assay.

Cell viability of essential oils after incubation with MCF-7 cell line

Initial results showed that essential oils had a clear effect on the MCF-7 cell lines from other cells, hence the focus was on this type in particular to complete the experiments compared to the other cells.

In this study, the percentage viability of MCF-7 cell line incubated with different concentrations of lemon, rosemary, and orange oil for 24 h were tested. The cell viability was decreased in a dose-dependent manner where the IC_{50} were 84 ± 1.3, 57 ± 2.8, and 59 ± 2.8 µl/ml for lemon, rosemary and orange oil respectively (Figure 2 and Table 1). ANOVA analysis of IC_{50} shows that the f-ratio

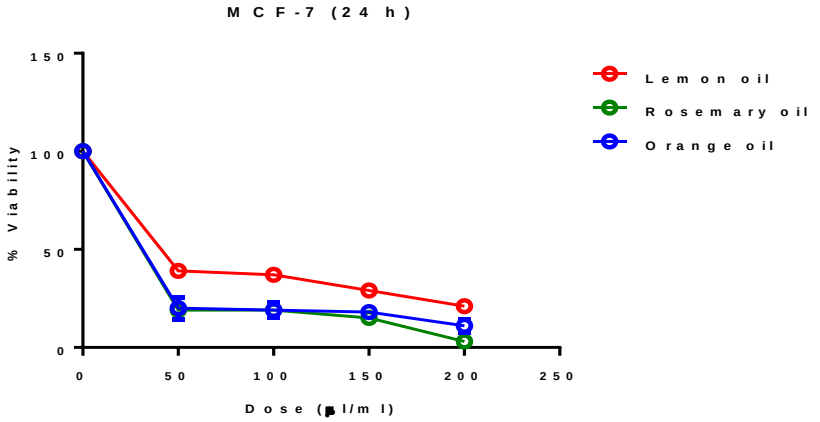

Figure 2. Percentage of cell viability of MCF-7 cancer cell line against different concentrations of lemon, rosemary, and orange oil measured 24 h after incubation. Results are presented as mean ± SEM (n=3).

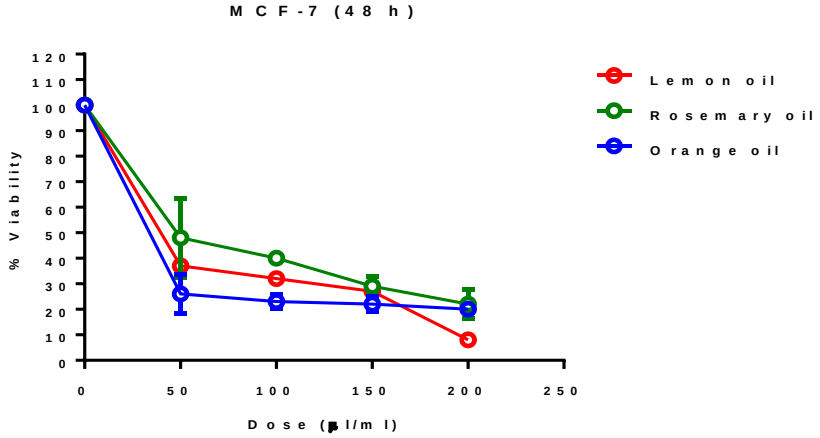

Figure 3. Percentage of cell viability of MCF-7 cancer cell line against different concentrations of lemon, rosemary, and orange oil measured 48 h after incubation. Results are presented as mean ± SEM (n=3).

value is 27.03666, the p-value is 0.000996, and the result is significant at $p \leq 0.05$. This indicates that both rosemary and orange oils are more effective than lemon oil (with p-value of 0.001943 and 0.002468). In addition, there is no significant difference between the effect of rosemary and orange at 24 h incubation period (p-value is 0.756019) (Table 1).

Cell viability after 48 h incubation with MCF-7 cell line

In MCF-7 cell line incubated with different concentrations of lemon, rosemary, and orange oil for 48 h, percentage viability was observed to decrease in a dose-dependent manner where the IC_{50} were 77 ± 1.9, 78 ± 10.6, and 69 ± 2.8 µl/ml for lemon, rosemary and orange oil respectively (Figure 3 and Table 1). ANOVA analysis of IC_{50} shows that the f-ratio value is 0.39042; the p-value is 0.692792; and the result is not significant at $p \leq 0.05$. This indicates that lemon, rosemary, and orange oils are nearly equally effective at 48 h incubation period (Table 1).

Cell viability after 72 h incubation with MCF-7 cell line

Finally, the percentage viability of MCF-7 cell line incubated with different concentrations of lemon, rosemary, and orange oil for 72 h was found to decrease in a dose-dependent manner where the IC_{50} was 94 ± 6.5, 63 ± 3.9 and 63 ± 2.5 µl/ml for lemon, rosemary and orange oil, respectively (Figure 4 and Table 4). ANOVA analysis of IC_{50} shows that the f-ratio value is 9.8061; the

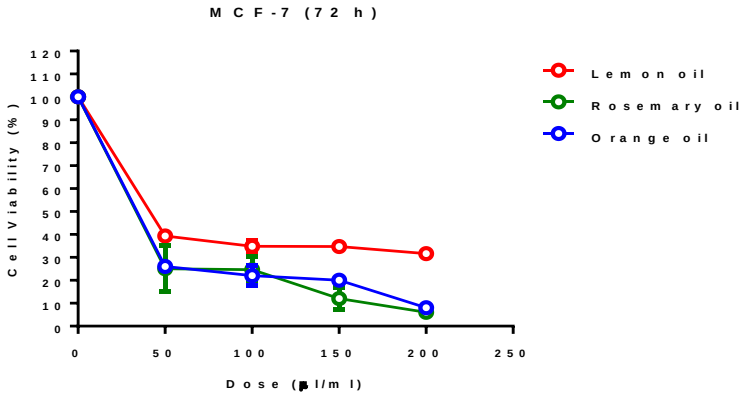

Figure 4. Percentage of cell viability of MCF-7 cancer cell line against different concentrations of lemon, rosemary, and orange oil measured 72 h after incubation. Results are presented as mean ± SEM (n=3).

Table 4. IC_{50} of lemon, rosemary, and orange oil measured 24, 48 and 72 h after incubation with MCF-7 cancer cell line.

Oil	IC_{50} (µl/ml) / MCF-7		
	24 h	48 h	72 h
Lemon oil	84 ± 1.3	77 ± 1.9	94 ± 6.5
Rosemary oil	57 ± 2.8[a]	78 ± 10.6	63 ± 3.9[a]
Orange oil	59 ± 2.8[a]	69 ± 2.8	63 ± 2.5[a]

Results are presented as mean ± SEM (n=3). [a] indicates significant difference compared to lemon oil group at p ≤ 0.05.

p-value is 0.012856; and the result is significant at p ≤ 0.05. This indicates that both rosemary and orange oil are more effective than lemon oil (p-value is 0.028979 and 0.023842). In addition, there is no significant difference between the effect of rosemary and orange at 72 h incubation period (with p-value of 0.902487) (Table 4).

In the present study, the constituents of the essential oils of rosemary, lemon and orange were identified to include monoterpenes, sesquiterpenes, alcohols and esters. Diethyl phthalate was demonstrated to account for the highest percentage of the components in lemon oil (60.61%).

A significant inhibitory effect was noted in the cell lines following treatment with the essential oils. This observation indicated that apoptosis may be a major contributor to the biological efficacy of the MCF-7 cells. The apoptosis rate was higher in the lemon essential oil group compared with that of other essential oil groups at three concentrations (P<0.01).

DISCUSSION

In lemon oil, the result is in consensus with previous studies of Rabi and Bishayee (2009) that identified D-Limonene, as one the components of lemon essential oil by using GC-MS. In this study, it was reported that D-Limonene can prevent human prostate cancer cells and enhance antioxidant activities. It is well known that the differences between the results of the present study and the chemical profile of previously investigated lemon oils are in the concentrations and types of the essential components which appeared to somewhat agree with some reports in Hamdan et al. (2013). Essential oils showed high variation in terms of composition even among closely related species (that is, species of the same genera), which is in agreement with previous reports on several plants (Sharma and Cannoo, 2013). A number of recent studies investigated the anticancer activities of several compounds. Lee et al. (2006) found that camphor suppresses the proliferation of cancer cells by inducing apoptosis. The diterpene alcohol, geranylgeraniol which was present in a relatively considerable amount (24.82%) in rosemary oil is reputed for broad spectrum of biological activities, as a potentially useful chemopreventive agent in hepatocarcinogenesis (Katuru et al., 2011). Among identified compounds, carboxylic acids, dominated by n-Hexadecanoic acid and cis-9-hexadecanoic acid, constituted the largest group of

compounds. The higher content of carboxylic acids in the essential oil, compared to other groups of compounds, is an unusual finding, as most other essential oils reportedly contain terpenes and other hydrocarbons. The anticancer properties of monoterpenes are discovered every now and then. For instance, limonene, a monoterpene, has been shown to exhibit anticancer properties against different types of cancers (Gautam et al., 2014).

Using cancer cell apoptosis induction trials, previous studies have identified that specific components of essential oils are capable of inducing cancer cell apoptosis. For example, sesquiterpenes have anticancer activities that are likely to arrest the proliferation of prostate cancer cells (Shoemaker et al., 2005; Sharifi-Rad et al., 2017). In addition, geranyl acetate and geranylgeraniol are two important monoterpenes found in the essential oils of several plant species (Qi et al., 2018; Dosoky and Setzer, 2018).

Geranylgeraniol have been shown to be of tremendous pharmacological potential activity. Moreover, previous studies have identified antitumor activity in two compounds with slightly greater contents of volatile oil, D-limonene, and n-Octanol (Zhang et al., 2017; Sun et al., 2010). However, the activities and mechanisms of specific compositions must be investigated in future studies.

Conclusion

From the EOs tested in this study, it was noticed that some had potential activities against the cancer cell tested. Factors such as processing, handling, and storage conditions may be responsible for the faintness in the biological and pharmacological properties of the commercial EOs, which originally possess significant biological activities, suggesting that more investigation on commercial Eos is required before recommending their use to the public.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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