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Full Length Research Paper

Analysis of the mitotic effect of *Annona muricata* leaf extracts on *Allium cepa* root tip cells

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There has been a growing interest in medicinal plants by the locals of the Pacific Islands since it is readily available. *Annona muricata*, commonly called soursop is one of such plants which is popular because it is being used to treat initial stages of cancer due to its antioxidant properties. This study investigated various extraction methods which could be used to compare and see the effectiveness of each on the chromosomes. The cytotoxicity and genotoxicity effect were studied using *Allium cepa* root tip cells. Eight onion bulbs were subjected to the treatment groups which were made using methanol, water and hexane extracts of *A. muricata*. Three different concentrations from each extraction solvent were used. The *A. cepa* root tip cells were subjected for 24 h to three different leaf extractions after left to grow in water. Concentrations of 12.5, 25 and 50 g/L were used. After the treatment, onion bulbs were put back in water to recover. Hence, 5 root tips from each bulb were removed (before treatment, treatment and recovery) with cells undergoing mitosis counted under 400x of a compound microscope. All studied concentrations of *A. muricata* methanol extract showed significant ($p \leq 0.05$) drop in the mean mitotic index when compared with control (mean MI reduced by 9.9, 44.4 and 54.2% for 12.5, 25 and 50 g/L respectively). *A. muricata* water extract also showed significant differences at 12.5 g/L where mitotic index (MI) was reduced by 8.6%. At 25 g/L, the reduction was by 35.3% and by 42.8% at 50 g/L. Drastic effect ($p \leq 0.05$) was observed with the soursop-hexane extract that reduced the MI significantly in 12.5 g/L by 5.0% and by 29.7% at 25 g/L. A huge reduction in MI was observed at 50 g/L where MI declined by 72.8%. Chromosomal aberrations were also observed which indicates the genotoxicity of the extracts. All the extraction methods contributed significantly towards the drop in total MI. Compounds in the extracts is mitodepressive.

Key words: *Allium cepa*, *Annona muricata*, chromosome aberration, cytotoxicity, mitosis, mitotic index.

INTRODUCTION

The Melanesian countries have more diverse flora among all the countries in the South Pacific. For example in Fiji,

there are nearly 2500 reported vascular plants, 20% of which is used for medicinal purposes (Han, 1998). Even

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the medicinal system was enriched upon the arrival of Indians to Fiji since they introduced the herbal system that was being used in the Indian subcontinent (Han, 1998). Hence, there is an urgent need to study the benefits of these plants before the knowledge on its usage dies out (Mahmoud and Gairola, 2013).

Many remedies are common and are being used in all the tropical regions. For instance, the use of immature guava leaves is being used to treat diarrhoea (Han, 1998). Likewise, most of the common plants in the Pacific are being used to treat similar ailments. The popular herbal medicines are used as dressing on wounds or ointments to cure skin problems. Despite the influence of Western methods to solve health problems, the Islanders still have faith in herbal methods of curing by the healers of the natives (Han, 1998). However, recent civilization which brought about changes in lifestyle and developmental programs has decreased the ethno pharmacological information along with the tribal cultures (Kumar et al., 2015).

Moreover, epidemiological studies have suggested that the usual uptake of specific phyto-chemicals can lower the occurrence of certain types of cancer (Rahman and Khan, 2013). Medicinal plants richly contain herbal properties which have contributed in the development of new drugs including cancer with no adverse effects. Moreover, the consumption of fruits also prevents and slows down the onset of chronic degenerative diseases like cancer, hypertension and cardiovascular diseases (Okullo et al., 2014).

According to Raina et al. (2014), treatment of cancer by traditional medicine would be attaining a significant level to cancer research. The reason as to why medicinal plants have come under spotlight is due to the fact that it contains good immunomodulatory and antioxidant properties enabling these plants to be considered as anticancer drugs.

One common medicinal plant which have been associated with treatment of cancer is *Annona muricata* (soursop) (Biba et al., 2014). It is commonly referred to as seremaia, sarifa (Fiji), and apele (Tonga) (Han, 1998). *A. muricata* has been gaining popularity as an anti-cancer treatment due to reports being made of its cytotoxic activity (George et al., 2012).

The objectives were to determine: i) Which extraction method being methanol, hot water and hexane affected mitosis the most. Since plants contain both polar and non-polar compounds, and it is these compounds that play a major role in controlling cell division, it was necessary to use both types of solvents in order to compare which one would be more effective to extract the compounds dictating cell division strongly. Methanol and hot water (50°C) which are polar solvents and hexane being non-polar were used to extract compounds from the leaves of both plants; ii) Which concentrations of leaf extracts being either 12.5, 25 or 50 under different extraction methods reduced the mitotic index the most.

This gives a breakthrough as to which concentration starts to affect MI significantly.

Hence, three different extracts were used to investigate the effectiveness of each on reducing the cell division and the cytotoxic and genotoxic effect on the cells. It is also stated that the *Allium* test results are similar to mammalian tests (El-Shahaby et al., 2003).

MATERIALS AND METHODS

Preparation of the aqueous extracts

The plant leaves were collected locally around the Laucala Campus of The University of the South Pacific, Fiji with reference to Smith (1981) and a research officer from Koronivia Research Station, washed and dried at 50°C. Once the leaves were dry, it was pounded using mortar and pestle and stored in an air tight bag till further use (Bhat et al., 2013).

500 g of the dried powdered material was then soaked in 1000 ml of methanol (0.5 g/ml) for 7 days for extraction (Ping et al., 2012). The same procedure was repeated using water and hexane. The solvent was then evaporated to dryness using rotary evaporator after filtration. For methanol, temperature of 40°C was used (Tagne et al., 2014) since it evaporates well at this temperature, whereas for the hot water extraction, 60°C was used and 68°C for hexane. Stock solution was diluted with distilled water to 12.5, 25 and 50 mg/ml concentrations. Similar concentrations were used by Ratanavalachai et al. (2010). Magnetic stirrer was used to mix the extract homogenously with water.

Allium cepa root cap cells preparation

Onion bulbs (2n=16) were obtained from a local supermarket. The outer scales and the dry bottom plates of the bulbs were removed without destroying the root primordia. For each separate extract with certain concentration, a series of eight bulbs were placed in distilled water for 48 h to germinate. These pyrex glass beakers were covered with a black plastic to keep in dark (Akinboro et al., 2011) and placed in a room at 22°C.

After the newly emerged roots were 1-2 cm in length, the onion roots were treated with the specific leaf extracts for 24 h before it was returned to distilled water to recover for another 24 h.

This makes exposure of root tips to distilled water as negative control. Treatment is when after exposure to negative control root tips are put in extracts for 24 h and recovery is when after this treatment, the root tips are put back into distilled water for another 24 h.

Genotoxic effect

After 24 h under each exposure, 5 root tips were cut from each bulb (8 bulbs for each treatment method which makes it up to 40 root tips for each exposure), and returned for next step. The root tips were fixed in 3:1 (v/v) ethanol: glacial acetic acid and stored overnight at 4°C. They were placed in 70% (v/v) aqueous alcohol the next day and refrigerated until used. Five slides on average were made for each bulb for each treatment using root tips that were hydrolyzed in 1N HCl for 3 min. Stained root tips were squashed in acetocarmine stain. Each slide was viewed at 400x using a compound microscope to determine the MI, while photomicrographs were taken at x1000 for chromosomal aberration under oil immersion.

Analysis of cytotoxicity and genotoxicity

(i) The mitotic index (MI) was calculated per 1000 cells by using the ratio between the number of mitotic cells and the total number of cells scored and expressed as percentage (Balog, 1982; Sehgal et al., 2006; Al-Ahmadi, 2013).

(ii) Cytogenic effects were evaluated by considering chromatin aberrations like anaphase bridges, sticky metaphase and breaks of chromosomes (Akinsemolu et al., 2015).

Determination of total phenolic and flavonoid contents

The total phenolic content of the plant extracts was determined by the Folin-Ciocalteu method (Meda et al., 2005). According to Nassr-Allah et al. (2009), the method similar to the one that follows was used for seven medicinal plants of Egypt since medicinal plants which has antioxidant properties contains phenolic and flavonoid compounds.

Lyophilized powder of 0.1 g of the plant samples were dissolved in 1 ml of deionized water. 0.1 ml of this solution was mixed with 2.8 ml of deionized water, 2 ml of 2% (w/v) sodium carbonate, and 0.1 ml of 50% (v/v) Folin-Ciocalteu phenol reagent. After incubation for 30 min at room temperature, the relative absorbance of the reaction mixture was compared to deionized water with a UV spectrophotometer. Gallic acid was used as a standard phenolic to construct a seven point standard curve (0 to 200 mg/L), the total phenolic contents in plant extracts was determined in triplicate.

The aluminium chloride colorimetric method was used to determine the total flavonoid contents. Aliquots of 0.1 g of plant extracts were dissolved in 1 ml deionized water. 0.5 ml of this solution was mixed with 1.5 ml of 95% (v/v) alcohol, 0.1 ml of 10% (w/v) aluminium chloride hexahydrate, 0.1 ml of 1 M potassium acetate and 2.8 ml of deionized water. The absorbance was measured at 415 nm after incubation at room temperature for 40 min and compared to deionized water with a UV spectrophotometer. In order to construct a seven point standard curve, quercetin was chosen. Triplicated assays were used to determine the total flavonoid contents. The result was expressed as milligram quercetin equivalents (QE)/g dry weight of lyophilized powder.

Statistical analysis

MSTATc statistical software was used for analysis. Analysis of variance was used to compare the data on mitotic and phase indices to reassure the viability of the data and validity of results. Differences between the individual dosage group and the control of each extract was analyzed by means of the post hoc Tukey's HSD test of significance at $p \leq 0.05$ level.

RESULTS

Analyses of variance of the mitotic indices indicated significant differences ($p \leq 0.05$) between concentrations of soursop-methanol extract as far as inhibition effect and MI was concerned (Table 1). Overall decrease in MI was contributed to by all the stages of mitosis. MI was above 70% in control groups which decreased to 20.7% with 50 g/L soursop-methanol extract exposure. The recovery rate declined as the concentration of the extract increased.

A significant ($p \leq 0.05$) inhibition effect of all three concentrations of soursop-hot water extract on MI (Table

1) was observed. All phase of cell cycles contributed to the overall decrease in MI. In control group, the MI was above 70% which fell significantly to as low as 32.5% with 50 g/L soursop- hot water extract exposure. The cells gained the potential to divide when allowed to recovery in water; however, the potential decreased as the concentration increased.

Similarly, the study showed a significant ($p \leq 0.05$) reduction in the mitotic index of all three concentrations of soursop- hexane extract (Table 1). The reduction in the MI was significantly increased with increasing concentration of the extract. The overall decrease in MI was contributed to by all phases of cell cycles. The MI was above 70% in control group which dropped significantly ($p \leq 0.05$) to as low as 1% with 50 g/L soursop-hexane extract exposure. The recovery rate had relatively decreased as the concentration of the extract increased.

Cells showing chromosome laggards, bi-nucleated cell, prolonged bacilliform nucleus, prolonged nucleus, multi-polar anaphase, spindle disturbance in metaphase and anaphase bridge (Figure 1) were counted for each treatment group and mean percentage of cells are presented in Table 2.

The data as tabulated in Table 3 shows that phenolic content was highest in soursop-hexane extract (83.5 mg GAE/g dry weight). This was followed in descending order by extracts of soursop-methanol and soursop- hot water. The highest amount of flavonoid was extracted by soursop-methanol extract, that is, 380.3 mg of Quercetin/g dry powder.

DISCUSSION

Comparable results were obtained for the soursop-methanol extract (Table 1). There was significant difference in the mitotic index between the control and the different concentrations used since $p \leq 0.05$. Mitotic index dropped by 9.9% in 12.5 g/L. There was a huge reduction observed in 25 and 50 g/L where MI decreased by 44.4 and 54.2% respectively. According to Panda and Sahu (1985), a drop below 50% is called cytotoxic limit value and usually has sub lethal effects on the organism. This means that there had been a notable drop in the mitotic index and that the extract from soursop had interfered with the proteins in the cells hindering cell division.

As the concentration of soursop- methanol extract increased to 50 g/L, the ability of the cells to divide dropped drastically to 20.7%. This showed that at this concentration, the extract becomes cytotoxic. Similarly, a cytotoxicity study was conducted on MCF-7 cell line and its effect on expression of bcl-2 also using soursop-methanol extract leaves by Rachmani et al. (2012). As the concentration of the test material increased, the percentage of mean number of living cells MCF-7 cells

Table 1. Cytogenetic analysis (mean \pm SE; %) of *A. cepa* root tip cells exposed to different concentrations of soursop-methanol extract.

Treatment	Prophase	Metaphase	Anaphase	Telophase	Mitotic index
Soursop-methanol extract					
Control	71.33 ^a \pm 0.12	1.04 ^a \pm 0.02	0.80 ^a \pm 0.03	0.55 ^a \pm 0.03	73.72 ^a \pm 0.10
12.5g/L	63.77 ^b \pm 0.11	0.02 ^b \pm 0.01	0.01 ^b \pm 0.01	0.01 ^b \pm 0.01	63.81 ^b \pm 0.10
Recovery	69.66 ^c \pm 0.13	0.27 ^c \pm 0.01	0.21 ^c \pm 0.01	0.15 ^c \pm 0.01	70.29 ^c \pm 0.12
ANOVA ; <i>F</i> (2,117)	1125.41; <i>p</i> <0.000	1842.78; <i>p</i> <0.000	428.15; <i>p</i> <0.000	235.58; <i>p</i> <0.000	2194.38; <i>p</i> <0.000
Control	72.38 ^a \pm 0.16	0.95 ^a \pm 0.02	0.68 ^a \pm 0.03	0.48 ^a \pm 0.02	74.49 ^a \pm 0.15
25g/L	29.14 ^b \pm 0.09	0.40 ^b \pm 0.02	0.32 ^b \pm 0.02	0.22 ^b \pm 0.01	30.08 ^b \pm 0.10
Recovery	39.79 ^c \pm 0.10	0.00 ^c	0.00 ^c	0.00 ^c	39.79 ^c \pm 0.10
ANOVA <i>F</i> (2,117)	35981.60; <i>p</i> <0.000	778.72; <i>p</i> <0.000	356.04; <i>p</i> <0.000	240.66; <i>p</i> <0.000	39346.13, <i>p</i> <0.000
Control	72.55 ^a \pm 0.15	1.01 ^a \pm 0.02	0.77 ^a \pm 0.03	0.56 ^a \pm 0.03	74.89 ^a \pm 0.14
50g/L	20.65 ^b \pm 0.12	0.00 ^b	0.00 ^b	0.00 ^b	20.68 ^b \pm 0.12
Recovery	29.62 ^c \pm 0.12	0.00 ^b	0.00 ^b	0.00 ^b	29.63 ^c \pm 0.12
ANOVA <i>F</i> (2,117)	45126.86; <i>p</i> <0.000	2502.13; <i>p</i> <0.000	712.53; <i>p</i> <0.000	418.79; <i>p</i> <0.000	52980.16; <i>p</i> <0.000
Soursop-hot water extract					
Control	73.19 ^a \pm 0.09	1.07 ^a \pm 0.02	0.98 ^a \pm 0.02	0.88 ^a \pm 0.03	76.11 ^a \pm 0.082
12.5g/L	67.55 ^b \pm 0.11	0.00 ^b	0.00 ^b	0.00 ^b	67.55 ^b \pm 0.11
Recovery	74.23 ^c \pm 0.23	0.89 ^c \pm 0.02	0.89 ^c \pm 0.03	0.72 ^c \pm 0.03	76.64 ^c \pm 0.21
ANOVA <i>F</i> (2,117)	514.27; <i>p</i> <0.000	1180.72; <i>p</i> <0.000	735.50; <i>p</i> <0.000	378.22; <i>p</i> <0.000	1225.16; <i>p</i> <0.000
Control	72.51 ^a \pm 0.61	1.16 ^a \pm 0.02	1.01 ^a \pm 0.02	0.94 ^a \pm 0.03	75.62 ^a \pm 0.64
25g/L	40.32 ^b \pm 0.65	0.00 ^b	0.00 ^b	0.00 ^b	40.32 ^b \pm 0.65
Recovery	59.81 ^c \pm 0.18	0.74 ^c \pm 0.04	0.50 ^c \pm 0.04	0.28 ^c \pm 0.03	61.35 ^c \pm 0.20
ANOVA <i>F</i> (2,117)	949.42; <i>p</i> <0.000	661.84; <i>p</i> <0.000	426.81; <i>p</i> <0.000	419.72; <i>p</i> <0.000	1087.04; <i>p</i> <0.000
Control	72.38 ^a \pm 0.179	1.13 ^a \pm 0.018	1.03 ^a \pm 0.025	0.87 ^a \pm 0.020	75.36 ^a \pm 0.166
50g/L	32.51 ^b \pm 0.66	0.00 ^b	0.00 ^b	0.00 ^b	32.53 ^b \pm 0.66
Recovery	55.03 ^c \pm 0.31	0.18 ^c \pm 0.02	0.15 ^c \pm 0.01	0.09 ^c \pm 0.01	55.45 ^c \pm 0.31
ANOVA <i>F</i> (2,117)	2141.56; <i>p</i> <0.000	1942.97; <i>p</i> <0.000	1092.57; <i>p</i> <0.000	1311.97; <i>p</i> <0.000	2496.35; <i>p</i> <0.000
Soursop-hexane extract					
Control	70.60 ^a \pm 0.10	1.14 ^a \pm 0.02	1.03 ^a \pm 0.02	0.84 ^a \pm 0.02	73.61 ^a \pm 0.09
12.5g/L	67.20 ^b \pm 0.21	0.64 ^b \pm 0.03	0.45 ^b \pm 0.02	0.34 ^b \pm 0.02	68.65 ^b \pm 0.18
Recovery	70.88 ^a \pm 0.22	1.09 ^a \pm 0.02	0.90 ^c \pm 0.02	0.72 ^c \pm 0.03	73.59 ^a \pm 0.20
ANOVA <i>F</i> (2,117)	124.80; <i>p</i> <0.000	171.13; <i>p</i> <0.000	217.31; <i>p</i> <0.000	134.31; <i>p</i> <0.000	309.88; <i>p</i> <0.000
Control	70.98 ^a \pm 0.11	1.09 ^a \pm 0.02	0.92 ^a \pm 0.02	0.69 ^a \pm 0.03	73.68 ^a \pm 0.09
25g/L	43.60 ^b \pm 0.30	0.19 ^b \pm 0.02	0.01 ^b \pm 0.00	0.06 ^b \pm 0.01	44.00 ^b \pm 0.29
Recovery	50.27 ^c \pm 0.15	0.43 ^c \pm 0.02	0.35 ^c \pm 0.02	0.18 ^c \pm 0.01	51.24 ^c \pm 0.16
ANOVA <i>F</i> (2,117)	4959.78; <i>p</i> <0.000	834.89; <i>p</i> <0.000	467.88; <i>p</i> <0.000	365.33; <i>p</i> <0.000	6017.98; <i>p</i> <0.000
Control	71.38 ^a \pm 0.15	1.06 ^a \pm 0.02	0.79 ^a \pm 0.03	0.64 ^a \pm 0.02	73.87 ^a \pm 0.14
50g/L	1.09 ^b \pm 0.08	0.00 ^b	0.00 ^b	0.00 ^b	1.09 ^b \pm 0.08
Recovery	9.36 ^c \pm 0.09	0.30 ^c \pm 0.02	0.19 ^c \pm 0.01	0.11 ^c \pm 0.01	9.96 ^c \pm 0.09
ANOVA <i>F</i> (2,117)	116059.32; <i>p</i> <0.000	969.12; <i>p</i> <0.000	661.99; <i>p</i> <0.000	566.94; <i>p</i> <0.000	143213.11; <i>p</i> <0.000

For each treatment, the same letters do not differ significantly at 0.05 level.

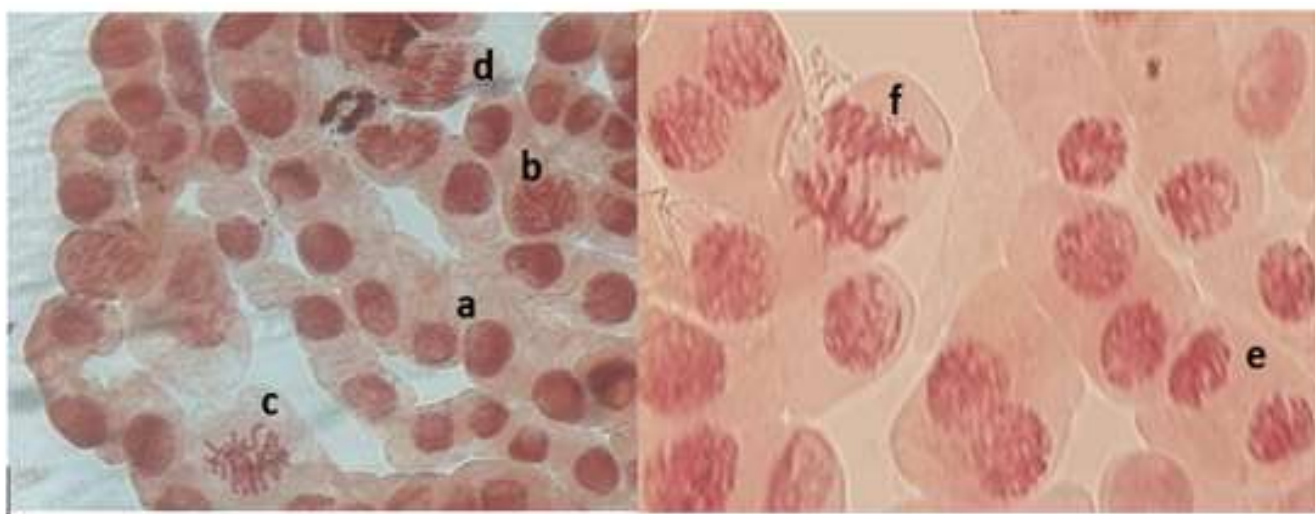
Table 2. Abnormal cells (mean \pm SE; %) of *A. cepa* root tips exposed to different extracts and concentrations of soursop.

Treatments	Methanol	Hot water	Hexane
Control	0.00 \pm 0.00	0.00 \pm 0.00	0.00 ^a \pm 0.00
12.5g/L	0.00 \pm 0.00	0.00 \pm 0.00	0.20 ^b \pm 0.07
Recovery	0.00 \pm 0.00	0.00 \pm 0.00	0.00 ^a \pm 0.00
ANOVA <i>F</i> (2,117)	-	-	7.429; p= 0.001
Control	0.00 \pm 0.00	0.00 ^a \pm 0.00	0.00 ^a \pm 0.00
25 g/L	0.00 \pm 0.00	0.00 ^a \pm 0.00	0.15 ^b \pm 0.06
Recovery	0.00 \pm 0.00	0.20 ^b \pm 0.07	0.13 ^c \pm 0.05
ANOVA <i>F</i> (2,117)	-	7.43; p= 0.001	3.19; p= 0.045
Control	0.00 ^a \pm 0.00	0.00 ^a \pm 0.00	0.00 \pm 0.00
50g/L	0.25 ^b \pm 0.10	0.13 ^b \pm 0.05	0.00 \pm 0.00
Recovery	0.08 ^a \pm 0.04	0.00 ^a \pm 0.00	0.00 \pm 0.00
ANOVA <i>F</i> (2,117)	4.13; p= 0.017	5.57; p= 0.005	-

For each treatment, the same letters do not differ at 0.05 level.

Table 3. Total phenolic and flavonoid content of different crude extracts from the leaves of soursop

Crude extracts	Total phenol(mg of GAE/g dry powder)	Total flavonoids(mg of Quercetin/g dry powder)
Soursop-hot water	78.5	68.2
Soursop-methanol	83.0	380.3
Soursop-hexane	83.5	66.0

**Figure 1.** Stages of mitosis as observed under 400 x. (a-e) Normal stages of cell division: (a) interphase; (b) prophase; (c) metaphase; (d) anaphase; (e) telophase; (f) anaphase bridge.

was reducing. At the least concentration of 31.25 mg/mL, the average percentage of cell survival was 75.3, whereas 5.3% was the cell survival rate at 50 mg/mL.

Hence they concluded that soursop- methanol leaf extract shows cytotoxicity. Results obtained by Raybaudi-Massilia et al. (2015) also demonstrated that methanol

extract of soursop seeds had significant cytotoxic effect ($p \leq 0.05$) on two human tumor cell lines it being HeLa and PC3.

For soursop-hot water extract, the mitotic index was significantly different from control, 12.5 g/L and recovery. This means that even at a lower concentration, this extract was able to reduce the mitotic index significantly by 8.6%. Once put back in distilled water to recover, mitosis rate increased with a significant difference at $p \leq 0.05$. Moreover, there was a significant ($p \leq 0.05$) drop by 5.6, 1.07, 0.98 and 0.88% for individual stages like prophase, metaphase, anaphase and telophase respectively. All these stages contributed towards an overall drop in the MI since division of the cells was slowed down and also hindered.

When the concentration was increased to 25 g/L, there was a significant drop in mitotic index by 35.3%. Mitotic stages like prophase, metaphase, anaphase and telophase recorded a significant ($p \leq 0.05$) drop of 32.2, 1.16, 1.01 and 0.94% respectively. When allowed to recover in water, it showed a marked increase in the mitotic index although not being able to divide fast enough to match the control's mitotic index. This means that at higher concentrations, recovery period of more than 24 h would be needed in order for the cells to reach its full capability of cell division.

The highest concentration (50 g/L) recorded the lowest mitotic index. Division of cells dropped significantly ($p \leq 0.05$) by 42.8% (Table 1). This could be the most toxic dose concentration since it hugely affected the division of cells. Recovery with water was possible; however, more than 24 h is essential for the cells to return to its full potential of dividing. Prophase decreased by 39.9%, metaphase by 1.13%, anaphase by 1.03% and telophase by 0.87% significantly ($p \leq 0.05$).

It has also been proven that the *in vitro* activity of antioxidant by soursop-water extract has significant antioxidant activity, hence can manage diseases by reducing oxidative stress (Gavamukulya et al., 2014).

The results obtained from this experiment were similar to the previous studies on *A. muricata* by Diaz et al. (2016). *A. cepa* cells that were treated with 1, 3, 5 and 6 and 7% of *A. muricata* -hot water extract showed differences in the mitotic index obtained. Out of the five extracts used, 1% extract showed the highest MI of 85.59% whereas 7% concentration showed the lowest index of 7.82%. This decrease in the MI as concentration increased is due to the cytotoxic compounds.

Soursop-hexane extract showed a marked potential in reducing MI especially at higher concentration. (Table 1). When the bulbs were exposed to 12.5 g/L, there was a difference by 5.0% in the reduction of division of cells from the control. This showed that even at the lowest concentration, soursop-hexane extract does have the potential in affecting the proteins in the cells. However, when the root tips were put back in water to recovery, it recovered by 4.9% and reached to a level within 24 h

where there was no significant difference between the control and recovery ($p \leq 0.05$). Prophase, metaphase, anaphase and telophase stages dropped significantly by 3.4, 0.5, 0.58 and 0.5%, respectively.

As the concentration was increased to 25 g/L, there was more hindrance to mitosis by 29.7%. A drop in the percentage of prophase was by 27.3%, metaphase by 0.9%, anaphase by 0.91% and telophase was by 0.63%. Recovery was significantly high when compared to the treatment group (increased by 7.24%). However, since the root tips were exposed to a higher concentration, recovery was not able to reach the fullest since the MI was significantly different and lower in the recovery group to the control.

Further increase in concentration to 50 g/L caused a drastic effect on the cells. It reduced the mitotic index by 72.8% which could have been the most toxic dose for the cells. There was cessation of all the mitotic stages except for prophase. When allowed to recover, there was significant ($p \leq 0.05$) difference recorded for cell division; however, it was still away by 63.9% to reach the full potential of cell division and may have needed more than 48 h to reach normality (Table 1). There was a drop in prophase by 70.30%, metaphase by 1.06%, anaphase by 0.79% and telophase by 0.64%. All stages of mitosis contributed significantly towards the total MI.

The ability of the soursop- hexane extract to inhibit mitosis was due to the fact that it contains compounds restricting the cells to divide. A research study conducted by Rosdi et al. (2015) found out that *A. muricata* -hexane leaves extract consist of flavonoids which played a role in decreasing cell viability in pancreatic cancer cells of humans (Capan-1) *in vitro*. Hence they concluded that this leaf might have anti-cancer agent. It has also been reported by Haro et al. (2014) after the characterisation and phytochemical screening of soursop leaves that it contains tannins, saponins, glycosides, alkaloids, flavonoids and steroids/triterpenoids.

Alongside this, it was also found out by Ningsih et al. (2015) that as the concentration of *A. muricata*-hexane extract increased, the inhibition zone diameter against *P. acnes* got bigger. Similarly, antibacterial activity decreased with decreasing concentration. Hence it was concluded that as concentration of the extract increased, its inhibition ability also increased.

Soursop crude extracts also have different levels of cytotoxicity towards breast cancer cell lines. The tumor's size and weight was reduced proving that soursop indeed plays a significant role in inhibiting cancer (Najmuddin et al., 2016).

The order of total phenol content was soursop- hexane > soursop -methanol > soursop-hot water. The highest flavonoid was detected in soursop-methanol extract (380.3 mg of Quercetin/g dry powder) and the least amount was found to be in soursop- hexane extract (66.0 mg of Quercetin/g of dry powder). The order of total flavonoids are soursop- methanol > soursop- hot water >

soursop-hexane (Table 3). Soursop-methanol extract did reduce the MI the most indicating the presence of most flavonoids which was the possible cause of hindrance to cell division.

Similar results were reported by Rantam et al. (2014) where they found that soursop leaves extract contained flavonoid from flavonol group which improved the activation and propagation of T-cell. Their result from immunocytochemistry for expression on rabbit PBMC culture showed that even the lowest dose of 5 µg of soursop leaf extract was able to arouse the expression of T-cell.

Cancer research regarding soursop has focused mainly on the phytochemicals known as Annonaceous acetogenins. These natural compounds are produced in the leaf, stem, bark and fruit seeds. From 37 species, 370 Annonaceous acetogenins have been obtained out of which 50% of over 80 of them were found to be significantly bioactive and could be fractionated. A rapidly growing class of compounds whose genuine anticancer ability as ATP inhibitors is currently being researched on. One of the very potent acetogenins is bullatacin which proves to be effective *in vivo* models (Kedari and Khan, 2014).

Moreover, few chromosomal aberrations were seen at all the three types of extracts (soursop-hexane extract at 12.5 and 25 g/L, soursop-methanol extract at 50 g/L and soursop-hot water extract at 50 g/L (Table 2). This could be due to chromosomal aberrations being observed mostly after prophase stage since *A. muricata* restricted most of the cells to proceed beyond prophase. Hence, only interphase and prophase stages were seen.

Conclusion

Hence, it can be said that *A. muricata* has compounds which affect the proteins in the cells and hinders cell division. The most hindrance was caused by soursop-hexane extract at the highest concentration which could be having the most compounds with antioxidant properties. The leaf extracts did contain significant amount of phenols and flavonoids. Further studies could be carried out using mammalian cancer cells to encourage its consumption as a medicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Quantitation of differential expression of transcription factors under moisture deficit stress in sugarcane

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Moisture deficit stress, one of the abiotic stresses, affects sugarcane growth and development and reduces cane and sugar yields. Transcription Factors (TFs) are master regulatory proteins in all living cells which have the capability of activating or repressing transcription of stress responsive genes in order to activate the stress tolerance mechanism. Study of expression profiles of TF genes which regulate the expression of stress responsive genes help to elucidate the regulatory biology of stress tolerance. Expression of 17 sugarcane TF genes in moisture deficit stress sensitive and tolerant varieties under different moisture deficit stress conditions were quantified in quantitative real-time PCR. Expression of seven TF genes namely, WRKY, NAC, bZIP, DREB, G2 like, Homeobox and TUB showed significant difference between the stress tolerant and susceptible varieties under both moderate and severe moisture deficit stress conditions. In stress tolerant variety, of these seven TF genes, bZIP showed highest expression both under moderate (22.39 fold) and severe stress (13.45 fold) conditions than other TF genes. Expression of bZIP gene in moisture deficit stress susceptible variety was significantly low under moderate (1.09 fold) and severe (3.63 fold) moisture deficit stress condition. GRAS TF gene under moderate stress condition (4 fold) and Homeobox gene under severe stress condition (6.06 fold) showed highest expressions than other TF genes in moisture stress susceptible variety. These differentially expressed TFs among the moisture stress tolerant and sensitive varieties hold promise for improving abiotic stress tolerance in sugarcane through their use as the potential candidate genes in marker assisted selection and in genetic transformation.

Key words: Transcription factors, moisture deficit stress, qRT- PCR, sugarcane.

INTRODUCTION

Sugarcane is, an important industrial crop, used primarily for production of sugar and ethanol. It is being cultivated in more than 100 tropical and subtropical countries

(Waclawovsky et al., 2010). Sugarcane is a highly water demanding crop and significant extent of existing growing areas cannot meet the water requirement during the most

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critical water demanding formative growth stage of the plant (Naidu, 1978; Ramesh, 2000). Supplementary irrigation is not a favorable option due to the added cost component which adversely affects the profit margin of the farmers. Hence, development of moisture deficit stress tolerant varieties either through conventional breeding or genetic manipulation is the most sustainable solution (Moore, 1987; Jain and Chattopadhyay, 2010) to mitigate the adverse effects comes from abiotic stresses.

Breeding for moisture deficit stress tolerance in sugarcane is complicated due to both the polyploid nature of the crop and the complexity of the trait (Swapna and Hemaprabha, 2010). Recent advancements in molecular tools help in identification of genetic factors involved in the responses of plants to moisture deficit stress. The available data indicate that moisture deficit stress tolerance is a complex physio-chemical process, in which many biological macro and micro molecules such as nucleic acids (DNA, RNA, microRNA), proteins, carbohydrates, lipids, hormones, ions, free radicals and mineral elements are involved (Bayoumi et al., 2008). Identification of genes which can regulate multiple biochemical and development pathways related to moisture deficit stress tolerance could be the best candidates to improve the performances of crops during moisture deficit stress. Among the stress responsive genes, TF genes are master regulatory proteins in all living cells. They often exhibit sequence specific DNA binding and are capable of activating or repressing transcription of multiple genes (Latchman, 2003). Interactions of TFs and *cis* elements in the promoter regions of stress responsive genes up-regulate the expression of many downstream genes and activate the stress tolerance mechanism (Agarwal and Jha, 2010). Hence, the ability of TFs to regulate the multiple biochemical and development pathways related to moisture deficit stress tolerance can be exploited to alter the performances of sugarcane during moisture deficit stress conditions. Information on the TFs which show differential expression under different moisture deficit stress conditions and quantification of the expression of TFs in susceptible and tolerant varieties are the prerequisites for understanding the regulatory biology of stress perception and modulation. This would help in identification of candidate genes which can be considered to target breeding for improved moisture deficit stress tolerance in sugarcane.

Hence, the present experiment was designed to identify the differentially expressed TFs and study the differences in their levels of expression under different moisture deficit stress conditions in sugarcane genotypes reported as moisture stress tolerant and sensitive.

MATERIALS AND METHODS

Two sugarcane varieties reported as moisture stress tolerant (Co 94008) (Gomathi and Vasantha, 2010) and moisture stress sensitive (Co 775) (Hemaprabha and Swapna, 2012; Manel and

Sumangala, 2017) were grown in pots filled with sterilized sand (single bud setts per pot) under greenhouse condition without light or temperature control. The experiment was arranged in a completely randomized design, with six biological replications for unstressed and stressed plants. All plantlets were maintained under same growth condition and equally watered (250 ml per pot) in alternate days with 25% of Hoagland solution per pot (Hoagland and Arnon, 1950). After 2 months of growth, four replications of each variety were subjected to moisture deficit stress by withholding the irrigation (stressed) and rest of the plants were regularly irrigated (unstressed). The water content of the sand was measured by gravimetric methods to observe the moisture reduction during the experimental treatment (Black, 1965). Root samples of two biological replicates of stressed and unstressed plants were collected separately 8 (moderate stress) and 10 days (severe stress) after withholding the irrigation (Rodrigues et al., 2009) and stored at -80°C.

Preparation of cDNA

Total RNA was isolated from stressed and unstressed root samples of each biological replicates separately using TRIzol reagent according to the instructions given by manufacture (SIGMA-ALDRICH PVT. LTD.USA). The quantity and quality of total RNA were checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and 1% formaldehyde Agarose gel electrophoresis. Single stranded cDNA was prepared by using High Capacity cDNA Reverse Transcription kit (cat#4374966, Ambion, USA) as per the protocol given by manufacturer.

The sequences of available forty-one sugarcane transcription factor families (http://grassius.org/browse_family.html/species=Sugarcane) were selected for quantification of expression. The similarity portion of the sequence of the members of TF families were identified and targeted for designing the primer pairs. The Primer3plus software (http://frodo.wi.mit.edu/cgi-bin/primer3plus/primer3plus_www.cgi) was used for designing the primer pairs and they were synthesized at Sigma-Aldrich Pvt. Ltd. (Germany) after confirming the specificity by BLAST searching them against SUEST database.

qRT-PCR and data analysis

Genomic DNA of sugarcane varieties, Co 94008 and Co 775 were used as the template and Polymerase Chain Reactions (PCR) were performed to confirm the applicability of the primers.

The Eppendorf master cycler realplex machine and its default program (95°C for 10 min, followed by 40 amplification cycles, 95°C for 15 s, 59-62°C for 20 s and 68°C for 20 s) were employed in 10 µl reaction mixture consisting of 1.0 ng cDNA, 200nM of each gene-specific primer and 5 µl of 2x SYBR green reagents (Cat.#4368706, Ambion, USA). Expression levels of TFs were quantified using $\Delta\Delta Ct$ method (Livak and Schmittgen, 2001). Sugarcane glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GAPDH F: 5' CACGGCCACTGGAAGCA 3' and GAPDH R: 5' TCCTCAGGGTTCCTGATGCC 3') reported as a stable gene in moisture stress studies (Ling et al., 2014) was used as the reference gene for normalization. Two each of technical and biological replications from each treatment were used to avoid the handling errors and to confirm the reproducibility of the results. Significant differences in gene quantitation between the stress and unstressed conditions and genotypes were analyzed on the basis of T-test sat $\alpha = 0.05$ using Microsoft Excel program.

RESULTS AND DISCUSSION

Phenotypic changes such as leaf rolling, wilting etc.

Table 1. Details of specific primers used for detection of transcription factors and amplicon size.

TF name	Forward primer sequence ^{5' - 3'}	Reverse primer sequence ^{5' - 3'}	T _m (°C)	Expected amplicon size (bp)	Observed amplicon size (bp)
ALFln like	GCCTCTGGTTGTCATATGTCTT	AAGGCCAAGAGGTTCCATTT	51.4	120	100 - 200
AP2 EREBD	TCAAGCAGCAGCAGAGGTAA	TCGGTGCTCATCTCCTTCTT	51.8	121	100 - 200
ARF	TGGGGAGTTACGTGTTGGAG	TGCAAGAACACCAAGATGCA	51.7	110	100
ARR-B	CCCAGCTTTGACCTCCCTG	CTCCTCGACGGTCATCTCC	55.4	191	100 - 200
BBR- BPC	CACTGGTGTGGTATGGTGG	ACCAAAGCCTAAGAAGCCTAAG	53.0	159	100 - 200
bZip	CAATGACCCTAGCAGACCCT	GTGGATTTGCAGCAACGGTA	52.8	153	100 - 200
E2F-DP	TCAAATTCGCCACACACA	GATGTCCCATAACGCCTAGCT	51.7	197	200
G2 -like	ATTCAGAAAGTACCGGCTGCA	TGCTCATCCATTTCCGCTTG	51.8	202	200
GRAS	ACTGTTCTGATGGCACCTGA	TCCCTTAGCCGTTTCTCTGG	52.8	209	200
GRF	ATCTCCCCTCCTCCCCTG	CAGGAAGGAGGATTGGGGAC	50.4	172	200
Homeobox	ACATGATCTGGGGCAACTGA	TACAGGCACAATTGGACCCT	51.8	165	100 - 200
MYB	GTTCCCTGCATGCTGAAACA	GGAACATTCACGGACACACC	52.8	233	200 - 300
TCP	GCTCATCCGTAACGCCAAG	CTGCTCGGACGGCTCAGT	54.1	116	100 - 200
TUB	AGATGTCTCGGCATGCTG	ACCTCTCCTCCTGATCCTCC	53.1	108	100
DREB	CACACAATCCAAGGGGCTTC	TGCCTCGTCTCCTTGAACTT	66.6	207	200
NAC	AAGTGAAAAGCTCCCCTCGA	TTTTCCCTCCTCTGGCTCTG	65.0	173	100 - 200
WRKY	GCGGGACCCCAGCTTCAAG	CCACGCCATGTCAAGCCGC	71.4	209	200

undergone by the moisture stress tolerant and sensitive varieties and reduction of the moisture content of sand potting medium during the experimental period were recorded (data not shown). A total of 17 primer pairs mentioned in Table 1 were designed to quantify the expression of TFs and their feasibility was checked in PCR with genomic DNA of the selected sugarcane varieties (Co 94008 and Co 775). Since all the primer pairs showed amplifications with genomic DNA (Plate 1a), their expressions were further quantified in qRT-PCR. Melting curve analyses for each primer pair were performed and confirmed the occurrence of specific amplification peaks and the absence of primer-dimer formation. Further, specificity of amplicons were confirmed by loading the qRT-PCR products on 4% Agarose gel and all

the selected TF genes showed the expected size of the amplicons (Plate 1b).

Differential expression of TFs in moisture stress tolerant and susceptible varieties

Differential expressions of TFs probably govern expression of stress-inducible genes either cooperatively or independently, and may constitute gene network in various responses for abiotic stresses (Joshi et al., 2016). Therefore, the TFs might play a role in the differential responses of moisture stress tolerant and susceptible varieties to moisture deficit stress. In this study, significant difference in expression of seven TF genes namely WRKY, NAC, bZIP, DREB, G2 like,

Homeobox and TUB were found between tolerant (Co 94008) and sensitive (Co 775) varieties under moderate and severe stress conditions (Table 2).

Among the differentially expressed TFs, bZIP showed the highest expression under moderate (22.39 fold expression) and severe (13.45 fold expression) stress condition. To date, several bZIP TFs have been functionally characterized, including those shown to be responsive to abiotic stress in rice (Lu et al., 2009) and *Arabidopsis* (Uno et al., 2000). For example, over-expression of OsbZIP23 or OsbZIP72 enhances the drought tolerance of rice and these two TFs are involved in ABA-dependent drought signal transduction (Lu et al., 2009). In this study also, higher expression of bZIP were recorded in moisture stress tolerant genotype than the sensitive genotype. The

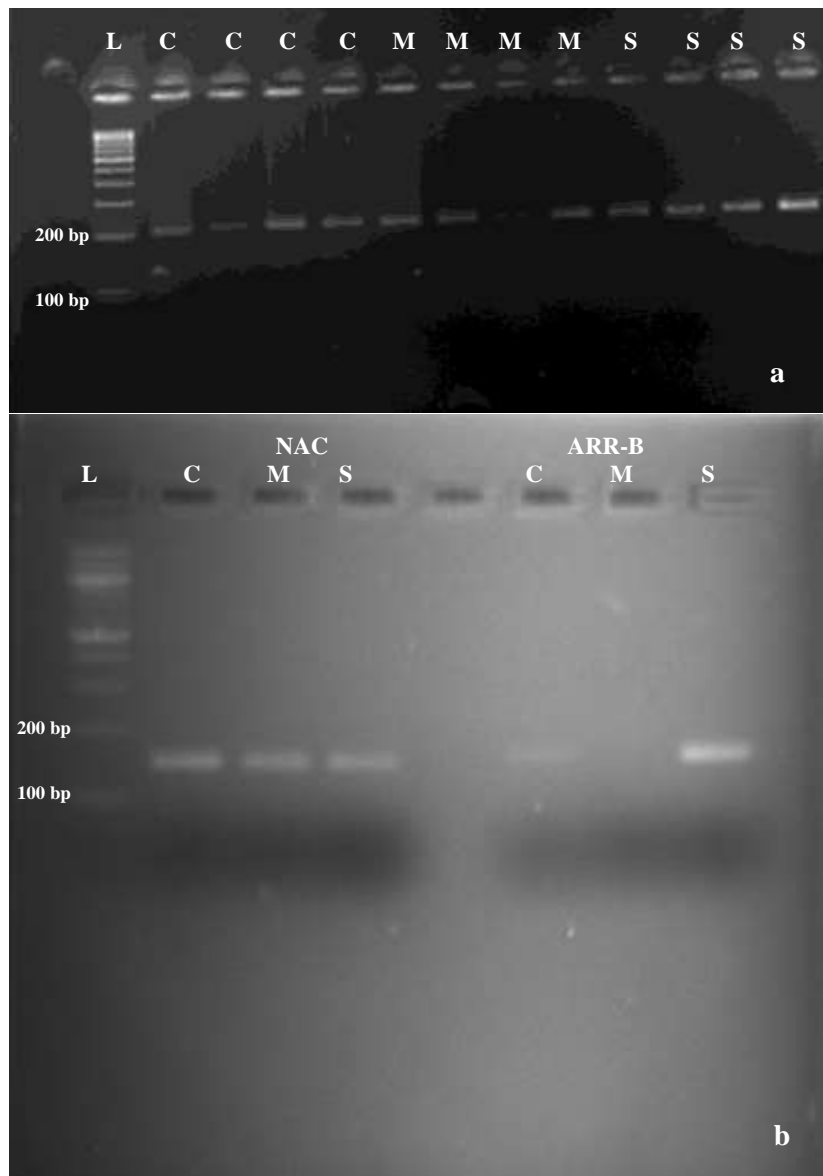


Plate 1. (A) Fractionation of PCR products generated from amplification of genomic DNA with the primers targeted the DREB gene, (B) Fractionation of qRT-PCR products generated from NAC and ARR-B primers on 4% agarose gel. L: Ladder, C: unstress condition, M: moderate stress condition and S: severe stress condition.

expression pattern of miRNA of bZIP factor in sugarcane indicated that tolerant plants adjust their transcriptome to increase the bZIP factor, which may activate the transcription of drought-related genes (Agustina et al., 2013).

Over-expression of the NAC transcription factor family members in *Arabidopsis* showed up regulation of several stress inducible genes in the transgenic plants and significant increase in drought tolerance (Tran et al., 2004). In the present study also, NAC TF gene showed significant up regulation than the unstressed condition in

both the sugarcane varieties (Figures 1 and 2). Further, expression of NAC gene was significantly high in moisture stress tolerant variety than susceptible variety.

DREB is a known ABA independent abiotic stress responsive TF that is expressed predominantly in moisture stressed root (Liu et al., 1998; Sakuma et al., 2002). Over expression of DREB gene and enhancement of moisture stress tolerance were reported in *Arabidopsis* (Liu et al., 1998), tobacco (Kasuga et al., 2004), rice (Oh et al., 2005) and potato (Behnam et al., 2006). Up-regulation of DREB TF in moisture stress tolerant

Table 2. Relative changes in the expression of transcription factors under stressed condition in Co 94008 and Co 775 sugarcane varieties

TF Family	Co 94008			Co 775			Calculated T value	
	Fold expression			Fold expression			M	S
	C	M	S	C	M	S		
GRAS	1	4	2	1	4	4	1.14	14.91**
GRF	1	0	0.06	1	0	0.03	UD	3.38*
G2-like	1	0.19	0.08	1	1.41	1.18	19.58**	12.09**
Homeobox	1	4	5.66	1	3.03	6.06	134.35**	6.44**
TUB	1	1	0.86	1	0.38	0.47	8.05**	6.97**
WRKY	1	1.4	0.68	1	3.4	1.01	135.19**	14.95**
NAC	1	4.12	1.94	1	1.43	1.33	66.03**	32.06**
bZIP	1	22.39	13.45	1	1.09	3.63	81.80**	211.31**
DREB	1	3.35	1.68	1	1.39	0.39	55.96**	56.08**

Table t value (5%, df: 3) = 3.18*, Table t value (1%, df: 3) = 5.84**
 Where; UD: Transcripts undetectable, C: Unstress condition, M: Moderate stress condition and S: Severe stress condition.

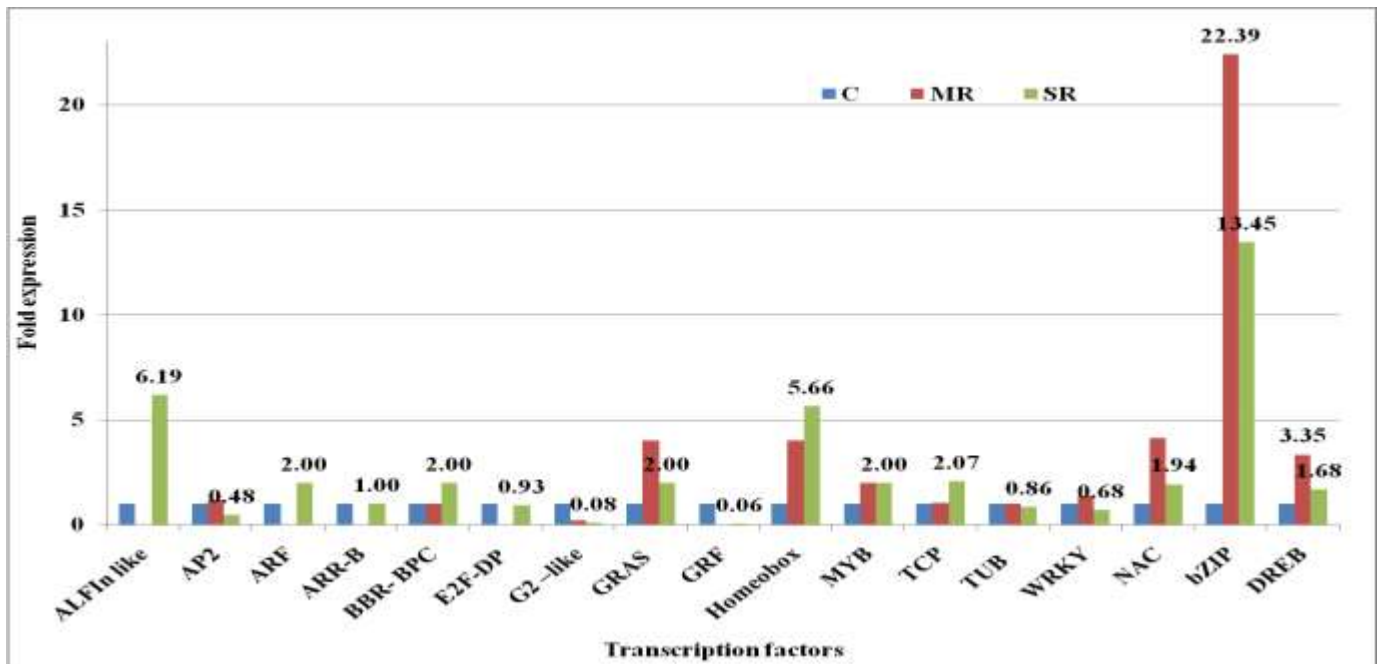


Figure 1. Real time PCR data for expression of transcription factors under stressed and control condition in sugarcane variety Co 94008, where: C: unstress condition, MR: moderate stress condition and SR: severe stress condition.

sugarcane varieties than sensitive was observed by Agustina et al. (2013) and the expression patterns of DREB in this experiment were also in accordance with the earlier reports. In this study also, DREB TF gene showed differential expression between sensitive and tolerant varieties under moisture deficit stress condition. Under moderate stress condition, it showed 3.35 fold up regulation in tolerant variety and 1.39 in sensitive variety. Though, this gene was down regulated under severe stress condition compared to moderate stress condition

in both varieties, expression in moisture stress tolerant variety Co 94008 showed higher expression than the sensitive variety. Hence, this gene may also contribute to moisture stress tolerance in Co 94008 variety.

Homeobox, a family of TFs are found only in plants and its over-expression increases tolerance to water stress (Dezar et al., 2005). Higher levels of expression of Homeobox were observed in tolerant genotype than sensitive genotype under moisture stress (Agustina et al., 2013). In the present study, up regulation of expressions

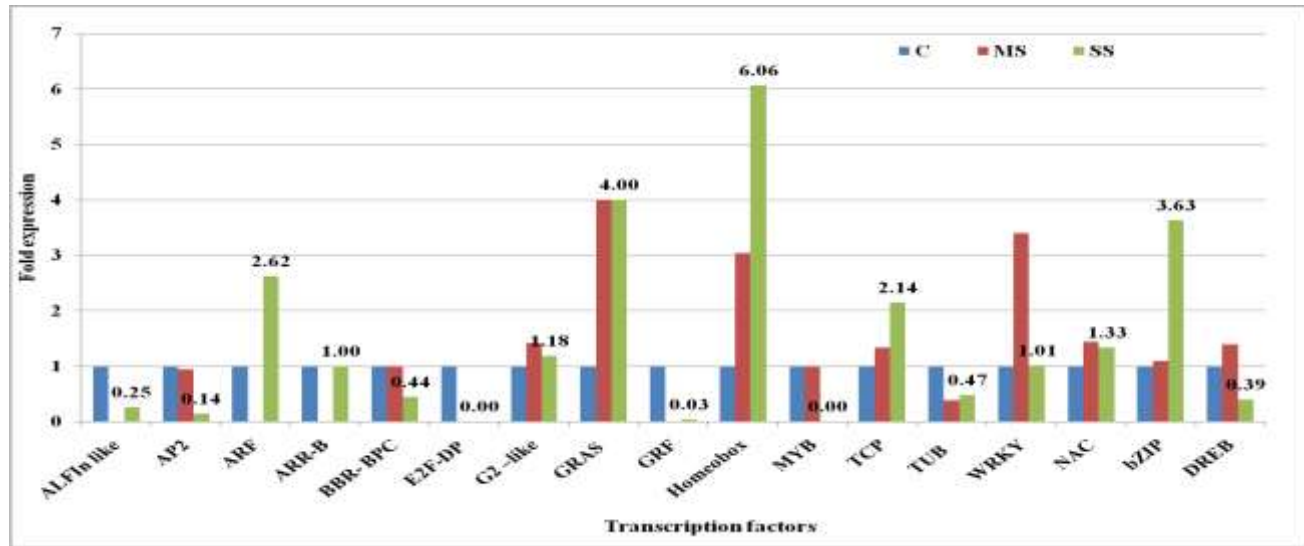


Figure 2. Real time PCR data for expression of transcription factors under stressed and control condition in sugarcane variety Co775, where: C: unstress condition, MS: moderate stress condition and SS: severe stress condition

of Homeobox TF was recorded for both the varieties under stress conditions and its expression were significantly different between the tolerant and sensitive variety (Table 2). Hence, Homeobox may also play a role in enhancing moisture stress tolerance in tolerant variety.

Several WRKY proteins were shown to be involved in plant drought and salinity stress responses in various species such as rice, tobacco and *Arabidopsis* (Golldack et al., 2011; Wu et al., 2009; Qiu and Yu 2009; Song et al., 2010). As an example, over-expression of OsWRKY11 under the control of HSP101 promoter led to enhanced drought tolerance, as shown by the slower leaf-wilting and increased survival rate of green plant parts (Wu et al., 2009). Further, previous research had demonstrated that WRKY proteins may act as activators or repressors of ABA stress hormone which plays essential role in plant responses to abiotic stress signaling (Chen et al., 2011). Ren et al. (2010) reported that over-expression of some WRKY proteins do not result in drought tolerance, thus they may need either co-factors or some posttranslational modifications to activate the downstream genes for stress tolerance. Recently, two research groups (Shang et al., 2010; Chen et al., 2010) reported the function of a group of structurally related WRKY proteins, in ABA signaling. Shang et al. (2010) showed that some WRKY proteins (WRKY40 in *Arabidopsis*) act as a central negative regulator among the WRKY proteins and could directly inhibit the expression of several important ABA responsive genes such as ABF, ABI, DREB, MYB and RAB, by directly binding to the W-Box sequences upstream of their promoters. Some WRKY genes (WRKY18 and WRKY60 in *Arabidopsis*) have a positive effect on plant ABA sensitivity and increase plant sensitivity to abiotic

stresses (Chen et al., 2010). In this experiment, WRKY gene showed the lower expression in tolerant variety (1.40 fold under moderate and 0.68 fold under severe stress) and higher in sensitive variety (3.40 fold under moderate and 1.01 fold under severe stress). Hence, it may enhance the sensitivity to moisture stress by playing the main role in ABA signaling.

Over-expression of the members of MYB TF family in different plant species showed increased tolerance to different abiotic stresses such as drought, chilling and freezing (Vannini et al., 2004; 2007; Pasquali et al., 2008). In the present study, MYB gene showed higher expression in tolerant genotype than sensitive genotype. MYB may act as enhancer of moisture stress tolerance. Further, expression of GRAS and GRF TF genes significantly differed between susceptible and tolerant varieties only under severe stress condition. The GRAS gene showed same level of expression in both varieties under moderate stress conditions (4 fold) and it was significantly low in Co 94008 (2 fold expression) than Co 775 (4 fold expression). These GRAS TFs are known to play a crucial role in diverse plant growth and development, ranging from gibberellic acid signaling, root radial patterning, light signal transduction and axillary shoot meristem formation (Hirsch and Oldroyd, 2009). Despite their important regulatory roles in *Arabidopsis*, the biological properties of GRAS members are largely unknown. One of GRAS TFs namely, OsGRAS23, has been identified in rice that is involved in drought stress response through regulating expression of stress-responsive genes (Xu et al., 2015). The functions of a number of identified GRAS genes and their role in moisture stress tolerance have not been characterized.

Expression of GRF TF gene under severe stress

condition was significantly low in Co 775 (0.03) than moisture stress tolerant Co 94008 (0.06) variety. The GRF transcription factors are involved in cell proliferation and strongly expressed in actively growing and developing tissues, such as shoot tips, flower buds, and roots, but weakly in mature stem and leaf tissues (Kim et al., 2003). Shunwu et al. (2012) reported that GRF TF was expressed under drought condition in rice. However, the functions of GRF genes related to drought resistance are unknown. Further studies on the functions of GRF genes will make obvious the role of this transcription factor in moisture stress tolerance.

Of the TFs which show significant differential expression between drought susceptible and tolerant varieties, expression of Homeobox showed increase with prolonged moisture deficit stress. Five TFs namely, GRAS, WRKY, NAC, bZIP and DREB showed up regulation of expression under moderate moisture deficit stress and down regulation under severe stress condition in tolerant variety Co 94008. It is presumed that the genes expressed during the course of gradual stress in tolerant species are responsible for altering the cellular metabolism, leading to adaptation under severe stress (Govind et al., 2009). These TFs genes may also provide the necessary induction to the plant to adapt and survive under severe stress.

Conclusion

Roots are the primary site of perception and injury, for several types of water limiting stresses including salinity and drought, in many circumstances; it is the stress sensitivity of the root that limits the productivity of the entire plant (Atkin et al., 1973; Steppuhn and Raney, 2005). Further, TFs are regulators of transcription and have the potential for coordinated regulation of genes relevant to stress tolerance (Xiong and Zhu, 2002). In this study, the bZIP TF gene out of the identified TFs: WRKY, NAC, bZIP, DREB, G2 like, Homeobox and TUB, reported from the analysis of Co 94008 and Co 775 varieties, may play an important role in moisture deficit stress tolerance in sugarcane. This information will be a valuable starting point for further research on these genes to check their potential as candidate genes to use as the targeted genes in moisture stress tolerance breeding programs. Further, the information generated may aid in isolation of most specific regulatory TFs and their promoters in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Comparison of oil quality extracted from selected conventional and non conventional sources of vegetable oil from Malawi

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In this study, oil quality with respect to physicochemical and phytochemical characteristics extracted from nonconventional seed oil namely *Moringa oleifera*, *Adansonia digitata*, *Parinari curatellifolia* and *Cajanus cajan* and conventional seed oil namely soybean (*Glycine max*) and groundnut (*Arachis hypogaea*) was assessed and compared. Results showed that there were significant differences in various quality parameters such as saponification number, peroxide value, free fatty acids in oils extracted from non-conventional and conventional sources. Oil yield ranged from 4.71 to 46.05% with pigeon peas registering the lowest yield and *P. curatellifolia* registering the highest yield. The following range of values in quality parameters were obtained: values in saponification number ranged from 55.91 mg KOH/g (*C. cajan*) to 220.54 mg KOH/g (groundnut): Peroxide value ranged from 2.79 meq O₂/kg (*M. oleifera*) to 10.47 meq O₂/kg (*C. cajan*): Free fatty acids ranged from 1.11 mg/100 g (*P. curatellifolia*) to 4.80 mg/100 g (pigeon peas): Specific gravity ranged from 0.87 (*P. curatellifolia*) to 0.91 (groundnuts): Oxalate ranged from 75.41 mg/100 g (groundnuts) to 632.56 mg / 100 g (pigeon peas): Acid value ranged from 2.21 mg KOH/g (*P. curatellifolia*) to 9.53 mg KOH/g (Pigeon pea): Iodine value ranged from 35.53 g/100 g (*P. curatellifolia*) and alkaloids ranged from 58.28 mg/g (*M. oleifera*) to 123.60 mg/g (groundnuts). Irrespective of the source of the oils, it was observed that the values in most of the oil quality parameters were within the acceptable levels. The findings in this study have demonstrated that nonconventional sources of oil have the potential to adequately supplement oils used for domestic consumption as well as industrial use in Malawi and therefore reduces the volumes of imported oils hence safeguarding foreign reserves.

Key words: Phytochemical, physicochemical, oil quality, *Moringa oleifera*, *Parinari curatellifolia*, *Adansonia digitata*.

INTRODUCTION

The world demand for fixed oils (vegetable oils and fats) is increasing with a consequential increase in prices (Mielke, 2017). Universally, vegetable oil consumption is

mainly based on soybean, palm, rapeseed and sunflower oil with 31.6, 30.5, 15.5 and 8.6 million tons consumed annually respectively (Stevenson et al., 2007). These

conventional sources of fixed oil fall short in meeting up the spiking demand of oil from both domestic and industrial sectors (Idouraine et al., 1996; Kojima et al., 2006).

Over the years, studies have been extensively carried out on the chemical composition of oil seeds of leguminous plants (Rusníková et al., 2013) and indigenous fruits (Abiodun et al., 2012; Edogbanya, 2016). Legume seeds play important role in human (Nwosu and Ojimekwe, 1993; Mbagwu et al., 2011) as well as animal nutrition contributing almost one-third of dietary protein (Graham and Vance, 2003; Rusníková et al., 2013). Legume seeds have been reported to have low fixed oil production with the exception of soybean (Rusníková et al., 2013) which has 22.7 ± 0.5 % oil content (Siulapwa and Mwambungu, 2014). On the other hand, pigeon pea (*Cajanus Cajan*) has been reported to contain 2.74% oil (Adebowale and Maliki, 2011) whereas groundnut (*Arachis hypogaea*) is reported to contain 39.10% oil (Kumar et al., 2013).

Indigenous tree seeds such as *Moringa oleifera* (Anwar et al., 2005), *Parinari curatellifolia* (Ndabikunze et al., 2006) and baobab (*Adansonia digitata*) seeds (Igboeldi et al., 1997; Abubakar et al., 2015) contain high amount of oil. Moringa is a domestic tree with various uses as food and medicine. It grows in the Middle East, tropics and subtropical areas of the world (Foidl et al., 2001). Moringa seed kernel contains 40% oil and 70% free fatty acids as oleic acids (Anwar et al., 2005). Moringa seed oil, commercially known as “Ben or Behen oil,” has properties that make it suitable for human consumption (Leone et al., 2016). Baobab (*A. digitata*) tree belongs to the Bombacaceae family and sub-family Malvaceae (Bremer et al., 2003; Osman, 2004) and is one of the underutilized crops in Africa (Temu et al., 2016). *A. digitata* seeds contain 12.20 ± 0.1 % (Osman, 2004) and 34.1 ± 0.2 (Ikemefuna and Amaechi, 1992; Ezeagu, 2005) oil.

P. curatellifolia, locally known as Maula in Malawi, belongs to the *Chrysobalanaceae* family (Oladimeji and Bello, 2011). It is a tropical evergreen tree that grows in sandy loam soil (FAO, 1982) and produces 47% oil from the nuts (Kernels) (Ndabikunze et al., 2006). Oilseeds have paramount importance in economics, nutrition and technology aspects. Oil produced from oilseeds is used in cooking, making soaps, cosmetics, lubricants, greases and agrochemicals (Idouraine et al., 1996; Nadeem and Imran, 2016). Fixed oils constitute part of our diet in supplying nutrients and energy to our bodies as well as flavor to our food (Atasie et al., 2009). Oils are sources of fat soluble vitamins like anti-oxidant vitamin E and protect sensitive or damaged cells from infections (Atasie et al., 2009). It is recommended that 40% of human energy

requirements should come from fats and oils besides nutrients provision (Sarwar et al., 2013).

Despite the main uses that oils possess, there is no single oil source that is suitable for all uses because of differences in their oil composition (Joshi et al., 2012). The quality of these oils for dietary purposes is based on parameters like acid value/free fatty acids, saponification values, peroxide value, and iodine value (Mousavi et al., 2012) besides the fractions of saturated and unsaturated fatty acid present in the oil (Rusníková et al., 2013). Based on this background, it is of paramount importance to intensify research on various aspects of oils such as in developing cooking oil supplies from non-conventional sources like Moringa seeds, *P. curatellifolia*, *A. digitata* and pigeon peas (*Cajanus cajan*). The objective of this current study was therefore to compare the oil quality parameters with respect to physicochemical and phytochemical characteristics in oils extracted from non-conventional and conventional sources of oil grown in Malawi.

MATERIALS AND METHODS

Sample collection and preparation

Moringa oleifera and *P. curatellifolia* seeds were obtained from communities surrounding Lilongwe University of Agriculture and Natural Resources, Bunda College Campus and Bunda forest respectively whereas *A. digitata* fruit seeds were bought from Mtchesi market in Lilongwe district. *A. digitata* seeds were washed in distilled water to separate the seeds from the pulp. Sundried woody *P. curatellifolia* seed stones, whose pulp were eaten by birds were collected from the ground below/underneath the trees. The seed kernels were removed from the woody seed stone by crushing the stones with a hard stone (Figure 1).

Soybean (*Glycine max*) seeds, pigeon pea (*C. cajan*) seeds and groundnuts (*Arachis hypogaea*) locally known as Nambwindi were bought from Mitundu local market, in Lilongwe district. *M. oleifera*, *Glycine max*, *C. cajan* and *A. hypogaea* (Nambwindi) seeds were manually sorted to remove dust, stones and those seeds infected by diseases (Olawumi et al., 2012). Dried samples were ground through a 1 mm sieve using a Thomas-WILEY model 4 Laboratory Mill before analyzing the physicochemical properties. The ground samples were used to analyze the qualities of crude fat using Association of Official Analytical Chemists (AOAC), 1996 methods with minor modifications.

Oil extraction procedure

Oil from the different samples was extracted by using petroleum ether in a soxhlet extractor / apparatus for 16 h. 20 g of finely ground sample was put into a porous thimble in a soxhlet apparatus connected to a weighed 250 ml flat bottomed quick fit flask containing 200 ml petroleum ether. The solvent was continuously boiled at 40 to 60°C extracting the fat from the sample. After 16 h of extraction the petroleum ether was evaporated by using a rotary

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Figure 1. Removing kernels from the stones.

evaporator. The flask containing the crude oil was then dried to constant weight at 105°C in the laboratory oven for 2 h. The crude oil was then refrigerated at 10°C in tight closed plastic bottles with no any further treatment waiting for some analysis (Adegbe et al., 2016).

Physicochemical analysis of oils

The determination of the physico-chemical properties of the oil followed the AOAC, 1996 methods with minor modifications.

Oil percentage

Following oil extraction method as described above, the oil percentage was calculated as in Equation 1.

$$\text{Oil \%} = \frac{(A-B)}{W} \tag{1}$$

Where: A= Weight of flask and oil after extraction (g)
 B= Weight of flask only (g)
 W = Weight of sample (g)

Determination of saponification value (SV)

1.0 g of the oil was weighed in a conical flask and 50 ml of 1.0 M ethanolic potassium hydroxide (KOH) was added. The flask was connected to a reflux condenser and was refluxed for 1 h until the solution became clear. A blank sample containing only 50 ml ethanolic Potassium hydroxide was similarly treated as the sample. The solution was then titrated to a faint pink colour end point against 1.0 M Hydrochloric acid (HCl) using phenolphthalein indicator (Ogungbenle and Sanusi, 2015). Saponification value (SV) was calculated as shown in Equation 2 below:

$$\text{SV (mg KOH / g)} = \frac{(A-B) \times N \times 56.02}{W} \tag{2}$$

Where: A= Blank ethanolic HCl volume in ml
 B= Sample ethanolic HCl volume in ml
 N= Normality of HCl, W=Weight of sample / oil in grams.

Determination of acid value (AV)

1.0 g of oil was weighed in a 250 ml conical flask containing 25 ml of absolute ethanol and diethyl ether (1:1) solution. The mixture was heated in a warm water bath (40°C) for 5 min and 3 drops of phenolphthalein indicator was added. The mixture was titrated against 0.1 M potassium hydroxide (KOH) to a faint pink color that persisted for 30 s. Acid value was then calculated as in Equation 3:

$$\text{Acid value (mg KOH/g oil)} = \frac{\text{ml (KOH)} \times N \times 56.1}{W} \tag{3}$$

Where N = Normality of KOH, W = Weight of oil sample in grams

Determination of free fatty acids (FFA)

Free fatty acids are the resultant of glycerin decomposition in oils and is measured as the number of milligrams of KOH required to neutralize a unit mass of oil. Therefore FFA value was analyzed by titrating 1.0 g of oil dissolved in 25 ml of absolute ethanol: diethyl ether (1:1 V/V) against 0.1 M ethanolic KOH to a faint pink color using phenolphthalein indicator. FFA is expressed as oleic acid equivalent and 0.1 M KOH = 28.2 g oleic acid as presented in Equation 4 (Okene and Evbuomwan 2014):

$$\text{FFA (g / 100 g as oleic acid)} = \frac{\text{Titre volume (ml) of KOH} \times 0.1 \text{ N} \times 28.2}{W} \tag{4}$$

Where N = Normality of ethanolic KOH, W = Weight of sample of oil in grams

Determination of peroxide value (PV)

1.0 g of oil sample was weighed into a 250 ml conical flask containing 20 ml of glacial acetic acid: chloroform solvent (3:2 v/v). 1.0 ml of saturated potassium hydroxide was then added to the mixture in the conical flask and was kept in the dark for 1 min. 30 ml of distilled water was added and the solution was titrated against 0.1 M Sodium thiosulphate (Na₂S₂O₃) solutions using 5 ml of starch as an indicator. A blank sample was treated as the samples. Equation 5 was used to obtain results which were expressed as meq per kilogram (Ogbunugafor et al., 2011).

$$PV \text{ (meq O}_2\text{/Kg)} = \frac{((V_2 - V_1) \times M) \times 1000}{W} \quad (5)$$

Where V_1 = Titre volume in ml of 0.1 M $\text{Na}_2\text{S}_2\text{O}_3$ for blank, V_2 = Titre volume in ml for sample, W = Weight of oil sample in grams.

Determination of iodine value (IV)

In determination of the iodine value (IV) of the oil, the methods described by the Association of Official Analytical Chemists (AOAC), 1996 and Choudhary and Pande (2000) methods were used with minor modifications in replacing carbon tetrachloride with cyclohexane.

0.5 g of oil was weighed in a 250 ml conical flask and 20 ml of cyclohexane: glacial acetic acid (1:1 V/V) solution was added into the flask. 10 ml of Wijs reagent was added to the flask, thoroughly mixed and kept in the dark for an hour. 15 ml and 100 ml of 15% Potassium iodide (KI) and distilled water were added to the flask and the solution was titrated against 0.1 M Sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution to colorless end point using starch as an indicator. The IV was calculated as shown in Equation 6:

$$IV \text{ (g I}_2\text{ / 100 g)} = \frac{(B - S) \times M \times 126.9 \times 100}{W \text{ (g)} \times 1000} \quad (6)$$

Where B = Volume 0.1 M Sodium thiosulphate used in titrating the blank

S = Volume of 0.1 M Sodium thiosulphate used in titrating the sample

126.9 = Molar mass of iodine

M = Molarity of Sodium thiosulphate

W = Sample weight in grams

Ester value (EV)

This is the milligrams of KOH that react with glycerin after saponification of a unit gram of oil. Therefore the EV was calculated as the different between the saponification value (SV) and acid value (AV) as shown in Equation 6.

$$\text{Ester value} = \text{saponification value} - \text{Acid value} \quad (7)$$

Determination of oil refractive index

Refractive index was measured using Bellingham and Stanley No.A83304 refractometer. A drop of oil was placed on the lower prism and the prism box was closed. The water flowed through the equipment jacket at 25°C, the light was adjusted then the compensator knob was moved to get a dark borderline on the cross wires which was viewed through the refraction view piece. The reading was recorded from the scale view through eyepiece (Ogungbenle, 2014).

Calculation of specific gravity

The specific gravity of the extracted oils was determined by calculation using the Lund equation as described by Halvorsen et al. (1993).

$$\text{Specific gravity} = 0.8475 + 0.0003SV + 0.00014IV \quad (8)$$

Where SV is saponification value and IV is iodine value.

Phytochemical chemical analysis of oils

Phytic acid determination

2 g of the sample was dissolved in 2% hydrochloric acid for 3 h and was filtered through Schleicher and Schuell 270 mm filter paper. 25 ml of the filtrate was mixed with 5 ml of ammonium thiocyanate and the mixture was titrated against 1.04% iron / Ferric chloride to brownish yellow color that persisted for 5 minutes (Reddy et al., 1992).

Alkaloids determination

5 g of the sample was dissolved in 20% Acetic acid in ethanol and the solution was left to stand for 4 h. The solution was filtered and was evaporated to one fourth of the solution. Concentrated ammonium hydroxide was added to the solution drop wise till precipitation was complete. The precipitate was filtered and dried in the drying oven to constant weight (Obadoni and Ochuko, 2001).

Oxalate determination

1 g of the sample was dissolved in 75 ml of 1.5 M Sulphuric acid and was stirred for 1 h and filtered. 25 ml of the filtrate was titrated while hot against 0.05 M potassium permanganate to a faint pink color that persisted for 30 s. Oxalate content was calculated as follows: 1 ml of 0.05 M KMnO_4 = 2.2 mg Oxalate (Chinma and Igyor, 2007).

Flavonoids determination

10 g of the sample was extracted with 300 ml of methanol: water (80:20 v/v) at room temperature for 1 h. The solution was filtered through a 125 mm whatman filter paper. The filtrate was transferred into a weighed crucible and evaporated to constant weight (Boham and Kocipai–Abyazan, 1974; Obadoni and Ochuko, 2001).

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 16. Analysis were done in triplicates and presented as means \pm SE. Analysis of Variance (ANOVA) with post-hoc was used to analyse and evaluate mean difference with a probability value of less than 0.05 being regarded as statically significant.

RESULTS AND DISCUSSION

Results on the physicochemical properties of the extracted oils are presented in Table 1.

Oil yield composition

Results on oil yield ranged from 46.05 \pm 0.19% to 4.71 \pm 0.12% for *P. curatellifolia* and *C. cajan* respectively. Values obtained for *M. oleifera* of 34.91 \pm 0.93% was slightly lower than the values of 38 and 45.8% reported by other researchers (Adegbe et al., 2016; Abiodun et al.,

Table 1. Physicochemical properties.

Parameter	<i>M. oleifera</i>	<i>P. curatellifolia</i>	<i>A. digitata</i> seeds	Pigeon pea (<i>C. cajan</i>)	Soybean (<i>G. max</i>)	Groundnut (<i>A. hypogaea</i>)
oil %	34.91±0.93 ^a	46.05±0.19 ^b	31.65±0.44 ^c	4.71± 0.12 ^d	19.32±0.36 ^e	20.70±0.28 ^f
Saponification value (mg KOH / g)	136.65± 0.14 ^a	90.09±0.30 ^b	122.54±0.11 ^c	55.91±0.06 ^d	55.93±0.02 ^e	220.54±1.76 ^f
Acid value as oleic (mg KOH / g)	9.46±0.02 ^a	2.21±0.01 ^b	2.21±0.01 ^b	9.53± 0.17 ^a	5.36±0.09 ^c	2.68±0.01 ^d
Peroxide value (meq O ₂ / Kg)	2.79±0.00 ^a	2.79±0.00 ^a	2.81±0.02 ^a	10.47± 0.12 ^b	5.18±0.21 ^c	2.85±0.06 ^a
Iodine value (g I ₂ / 100 g)	43.11±6.81 ^a	35.53±4.59 ^b	40.87±3.14 ^c	69.64± 5.19 ^d	52.23±3.95 ^d	53.30±6.54 ^e
Free Fatty Acids (mg / 100 g)	4.76±0.01 ^a	1.11± 0.00 ^b	1.11±0.00 ^b	4.80±0.09 ^c	2.63±0.05 ^d	1.35±0.01 ^e
Ester value (mg KOH / g)	127.18±0.13 ^a	127.18± 0.13 ^a	120.33±0.11 ^b	46.35± 0.15 ^c	50.69±0.07 ^d	217.86±1.75 ^e

For each parameter, means with same superscript were not significantly different ($P>0.05$).

2012). However, the value was in agreement with the work of other researchers in Sudan who reported a value of 34.8% (Anwar and Rashid, 2007). The oil content in *P. curatellifolia* kernel of 46.05±0.19% was found to be higher than 1.77% as compared to results obtained by other researchers (Ogungbenle and Atere, 2014) and 5.11±0.10% (Oladimeji and Bello, 2011) reported in Nigeria. However, it was closely similar to the value of 47% which was reported by other researchers (Ndabikunze et al., 2006) for studies conducted in Tanzania. On the other hand, *A. hypogaea* and *G. max* had similar oil yields of 20.70±0.28% and 19.32±0.36% which were lower than the value of 47.00±0.03% reported by other authors in a related study conducted in Nigeria (Atasie et al., 2009). However, the yield value of oil obtained in *A. digitata* seeds of 31.65±0.44% was in agreement with the value of 32±0.00 previously reported by other researchers (Abubakar et al., 2015).

Saponification value composition

Results showed differences in the values of

saponification value for the two sources of oil as well as values obtained by other researchers in previous studies. Groundnut (*A. hypogaea*) oil had the highest saponification value followed by *M. oleifera*, *A. digitata*, *P. curatellifolia*, *G. max* and *C. cajan* oils respectively. The saponification value of 220.54±1.76 mg KOH/g oil obtained in *A. hypogaea* was higher than 193.20 mg KOH/g (Atasie et al., 2009) for *A. hypogaea* oil obtained by other researchers in Nigeria. On the other hand, the saponification value in *M. oleifera* oil of 136.65±0.14 mg KOH/g was low compared to the value of 180.92 as reported previously by other researchers (Adegbe et al., 2016).

The saponification value for *P. curatellifolia* oil of 90.09±0.30 was lower than 135.1 reported in a study conducted in Zimbabwe (Ndaba, 2014).

Similarly, *A. digitata* seed oil saponification value of 122.54±0.11 was lower than 158.62±0.07 (Abubakar et al., 2015) as reported in Nigeria but higher than that of *G. max* and *C. cajan* oils obtained from this study. On the other hand, the saponification value of 55.93±0.02 for *G. max* was higher than 13.47±0.06 mg KOH/g (Essien et al., 2014). Interestingly, it was observed that the saponification values of crude oils were within the

recommended values of 180-199 mg KOH/g oil (FAO/WHO, 2009) for edible oil, 187-196 (FAO, 1995) and 189-195 mg KOH/g (FAO, 1995) for *A. hypogaea* and *G. max* oils. As reported by various authors, saponification value measures the oxidation state of the oil (Nkafamiya et al., 2010), type of fatty acids in oils (Adejumo et al., 2013) and average molecular weight of the oils (Preeti et al., 2007). The different oil quality characteristics that saponification value measures as well as the differences in the way the oils were processed and handled in the different countries probably might have explained the reasons for the differences in the values obtained in this study. The high saponification values of the oils indicate oxidative state of the oils and the low values indicate the onset of oxidation (Nkafamiya et al., 2010).

Acid value composition

Results showed that *C. cajan* and *M. oleifera* had the highest acid values of 9.53±0.17 and 9.46±0.02 mg KOH/g oil followed by *G. max* oil (5.36±0.09), *A. hypogaea* oil (2.68±0.01), *P. curatellifolia* (2.21±0.01) and *A. digitata*

(2.21±0.01). *A. hypogaea* acid value of 2.68±0.01 was lower than 5.99 mg KOH/g as reported by other researchers (Atasie et al., 2009) for studies done in Nigeria. Compared with values obtained from other studies, it has been observed that *M. oleifera* oil had acid value higher than 6.73 (Adegbe et al., 2016) and 7.09±0.21 (Abiodun et al., 2012) for studies conducted in Nigeria. The *A. digitata* acid value of 2.21±0.01 was found to be low as compared to 6.52±0.02 (Oyeleke et al., 2012) but was closely in agreement with 2.75±0.14 reported by Abubakar et al. (2015) in a related study conducted in Nigeria. *G. max* had acid value of 4.05±0.024 which was higher than the value reported by Okorie and Nwachukwu (2014). The acid values for *M. oleifera*, *C. cajan* and *G. max* oils obtained in this study were higher than the recommended value of 4.0 mg KOH/g oil for edible virgin and cold pressed oils whereas *P. curatellifolia*, *A. hypogaea* and *A. digitata* oil acid values were below the recommended standard of 4.0 mg KOH/g oil (FAO/WHO, 1999).

Acid value measures the degree of oil spoilage, in terms of free fatty acids (FFAs), from enzymatic activity (Amadi et al., 2013). This observation of high acid values in *M. oleifera*, *C. cajan* and *G. max* oil suggest that these oils contain higher levels of fatty acids, as oleic acids, than *P. curatellifolia*, *A. digitata* and *A. hypogaea*.

Peroxide value composition

Results on peroxide value similarly showed that there are differences in values for the two sources of oil. Peroxide values of crude oils ranged from 2.79±0.00 to 10.47±0.12 meq O₂/kg oil for *M. oleifera* and *P. curatellifolia* oils. The *A. hypogaea* peroxide value of 2.85±0.06 meq O₂/kg was closely similar to that of *A. digitata*, *P. curatellifolia* and *M. oleifera* oil registering 2.81±0.02, 2.79±0.00 and 2.79±0.00 meq O₂/kg oil respectively. When compared with values previously reported by other authors, it was observed that peroxide value in *M. oleifera* was similar to 2.60 meq O₂/kg (Adegbe et al., 2016) but higher than the value of 0.83±0.13 meq O₂/kg (Basuny and Al-Marzouq, 2016) and interestingly lower than the value of 15.96±0.13 meq O₂/kg (Abiodun et al., 2012) reported in studies conducted in Nigeria and Saudi Arabia. However, *A. digitata*, *P. curatellifolia* and *A. hypogaea* oils peroxide values were higher than the value of 1.5 (Atasie et al., 2009) for *A. hypogaea* oil reported in related studies. *G. max* had higher acid value of 5.18±0.21 than the value of 2.42±0.06 meq O₂/kg (Okorie and Nwachukwu, 2016) for *G. max* oil studies conducted in Nigeria.

The values obtained in *M. oleifera*, *A. digitata*, *G. max* and *P. curatellifolia* oils were found to be within the recommended value of 5.0 meq O₂/kg oil (FAO/WHO, 1999) for edible fat and oils whereas *C. cajan* peroxide value was within the recommended value of 10.0 meq O₂/kg oil (FAO/WHO, 1999) for edible virgin and cold

pressed fat and oils. Peroxide value measures the degree of either the occurrence of peroxidation or adulteration (Okene and Evbuomwan, 2014) and could be used to evaluate the quality and stability of oils during storage (Adejumo et al., 2013; Okene and Evbuomwan, 2014). Therefore the low peroxide values in *M. oleifera*, *P. curatellifolia*, *A. digitata* and *A. hypogaea* oils indicate that these oils are more saturated than *C. cajan* and *G. max* oils and therefore the low peroxide value reflects high quality in the oils.

Iodine value composition

Results showed that there were differences in iodine value composition for the two sources of vegetable oils. The iodine value for the extracted oils ranged from 35.53±4.59 to 69.64±5.19 g I₂/100 g for *P. curatellifolia* and *C. cajan* respectively. The iodine values for *M. oleifera* and *A. digitata* were very close as reflected by the recorded values of 43.11±6.81 and 40.87±3.14 respectively. On the other hand, *A. hypogaea* and *G. max* oils had similar iodine values of 53.30±6.54 and 52.23±3.95 which were lower than the value of 69.64±5.19 for *C. cajan* obtained in this study. When compared with findings from other authors, it was observed that the *M. oleifera* iodine value of 43.11±6.81 was lower than of 55.02±0.15 (Abiodun et al., 2012) and 68.41 (Siyanbola et al., 2015). On the other hand, *G. max* and *A. hypogaea* oil had low iodine values as compared to the values of 123.42 (Eze, 2012) and 38.71 (Atasie et al., 2009) for *G. max* and *A. hypogaea* reported in studies conducted in Nigeria. Similarly, values obtained in *A. digitata* revealed low iodine value of 40.87±3.14 as compared to the value of 54.41±0.94 (Abubakar et al., 2015) reported in studies conducted in Nigeria. The iodine values of the extracted crude oils were lower than the recommended iodine values of 90-115 g I₂/100 g oil for crude vegetable oil (FAO/WHO, 2009).

Iodine value measures the degree of unsaturation (number of double bonds) of the oils. The high iodine value reflects high degree of unsaturation (more double bonds) of the oils meaning that the oils easily undergo oxidation and rancidification reaction (Egbuonu et al., 2015). The low iodine values for the extracted oils observed in this study suggest that the oils are saturated and therefore have low susceptibility to oxidation and rancid reaction during storage.

Ester value composition

Results on ester value ranged from 46.35±0.15 to 217.86±1.750 mg KOH/g oil for *A. hypogaea* and *C. cajan* oils. Interestingly, *M. oleifera* and *P. curatellifolia* had similar ester values of 127.18±0.13 mg KOH/g oil which were higher than that of *A. digitata*, *C. cajan* and *G. max* but lower than 217.86±1.75 for *A. hypogaea* oil.

Free fatty acid composition

Results on free fatty acid composition showed that the values ranged from 1.11 ± 0.0 to 4.80 ± 0.09 mg KOH/g oil with *P. curatellifolia* and *A. digitata* registering the lowest values and *Moringa* and *C. cajan* the highest values. *A. hypogaea* free fatty acid value of 1.35 ± 0.01 was lower than the value of 3.01 mg KOH/g oil obtained by other researchers (Atasie et al., 2009) for work conducted in Nigeria. Contrastingly, the *M. oleifera* fatty acid value of 4.76 ± 0.01 was higher than the value of 2.8 and 3.3 (Anwar et al., 2006) for drought and irrigated *M. oleifera* and lower than the value of 11.2 for n-hexane extracted *Moringa* oil (Lalas and Tsaknis, 2002) obtained in related studies. The observed acid values for crude oils were higher than the recommended value of 0.6 mg KOH/g oil for refined edible oils (FAO/WHO, 1999).

Physical and phytochemical composition

The physical and phytochemical compositions of the extracted crude oils in mg/g oil are presented in Table 2.

Physical composition

Results on the refractive indices of the extracted oils ranged from 1.4627 ± 0.00 to 1.4695 ± 0.00 for *A. hypogaea*, *C. cajan* and *Glycine max* respectively. *A. digitata* had a refractive index of 1.4640 ± 0.00 which was slightly lower than the value of 1.4678 ± 0.00 for *M. oleifera* and *P. curatellifolia* oils respectively. The refractive index for *M. oleifera* oil was higher than the value of 1.4559 (Adegbe et al., 2016) but very close to the value of 1.4668 (Garba et al., 2015) as compared to studies previously done by other researchers. In addition, the *A. digitata* refractive index of 1.4640 ± 0.00 was similar to 1.498 ± 0.002 (Oyeleke et al., 2012) and 1.5 ± 0.0 (Osman, 2004) for *A. digitata* oil studies conducted in Nigeria and Saudi Arabia. Refractive indices of oils increase with either the increasing degree of unsaturation or increasing chain length of fatty acids in the triglycerides (Evans et al., 1974). The close values for the refractive indices of oils as presented in Table 2 probably suggest that the oils have either similar unsaturation or chain length. It was interesting to observe that the *A. hypogaea* and *A. digitata* refractive index values were within the recommended values of 1.460 - 1.465 (FAO/WHO, 1999) for *A. hypogaea* whereas the refractive indices for *P. curatellifolia* (1.4678 ± 0.0), *C. cajan* (1.4695 ± 0.0), *Glycine max* (1.4695 ± 0.0) and *M. oleifera* (1.4678 ± 0.0) were closely similar to the recommended value for crude *A. hypogaea* (FAO/WHO, 1999).

The calculated specific gravity for the crude oils ranged from 0.8650 ± 0.00 to 0.9144 ± 0.00 for *Glycine max* and *A.*

hypogaea respectively. *G. max* and *C. cajan* had similar specific gravity values of 0.8848 ± 0.0 and 0.8650 ± 0.00 whereas *P. curatellifolia*, *A. digitata* and *M. oleifera* had specific gravity values of 0.8750 ± 0.0 , 0.8848 ± 0.0 and 0.8891 ± 0.0 respectively. When compared with findings obtained by other researchers, it was observed that *M. oleifera* had lower specific gravity value than the value of 0.9050 (Adegbe et al., 2012) but was within the range of 0.91 ± 0.31 (Abiodun et al., 2012). On the other hand, *A. digitata* specific gravity value of 0.8848 ± 0.0 was close to the value of 0.9 ± 0.00 (Osman, 2004) but slightly lower than the value of 0.928 ± 0.001 (Oyeleke et al., 2012) for studies conducted in Saudi Arabia and Nigeria. The specific gravity values for the crude oils were within the recommended standard values of 0.9 - 1.16 for edible oils (FAO/WHO, 2009) and 0.919 - 0.925 for soybean oils (FAO, 1995).

Phytochemical composition

Results showed that the phytate composition for the extracted crude oils, in mg/g, ranged from 44.52 ± 0.40 to 240.47 ± 5.24 for *A. hypogaea* and *C. cajan* respectively. Phytate content in *M. oleifera* (87.78 ± 0.22) was low compared to *C. cajan* (240.47 ± 5.24) and *G. max* (104.32 ± 0.12) but higher than 65.25 ± 0.05 for *P. curatellifolia* and closely similar to the value of 86.08 ± 0.04 for *A. digitata* observed in this study. Phytic acid is an antinutritional factor that forms insoluble salts when mixed with food salts like phosphorus, calcium, iron magnesium and zinc making them unavailable for absorption into the blood system (Schlemmer et al., 2009). Phytate content in foodstuffs can be used in calculation of available phosphorus for absorption into the blood stream (Robert and Yudkin, 1999). Phytate content in *A. hypogaea* was high compared to the value of 4.18 mg/g oil obtained by other authors (Inuwa et al., 2011).

Oxalate content ranged from 75.41 ± 0.40 to 632.56 ± 4.90 mg/100 g for *A. hypogaea* and *C. cajan* respectively. Oxalate content for *A. digitata* was 210.08 ± 17.80 mg/100 g which was high compared to *M. oleifera* (149.916 ± 3.42 mg/100 g), *G. max* (116.04 ± 6.34), *P. curatellifolia* ($169.92(0.61)$ mg/100 g) and *A. hypogaea* but lower than *C. cajan* obtained in this study. Oxalate content in *A. hypogaea* was higher than the value of 4.18 mg/g oil (Inuwa et al., 2011) when compared with findings from other researchers. *M. oleifera* oil registered a higher oxalate content compared to the value of 4.12 ± 0.04 mg/g oil (Abiodun et al., 2012) reported in related studies.

Alkaloids content, in mg/g oil, ranged from 58.28 ± 4.88 to 1005 ± 1.0 mg/g for *M. oleifera* and *A. digitata* oil respectively. *P. curatellifolia* oil had alkaloid content of 102.65 ± 1.89 mg/g which was lower than the values obtained in *A. digitata* (1005 ± 1.0 mg/g), *G. max* (163.50 ± 3.80) and *A. hypogaea* (323.60 ± 23.84 mg/g) oil obtained in this study. Alkaloids contents in *G. max* and

Table 2. Physical and phytochemical properties of extracted oil.

Samples	Refractive index (25°C)	Specific gravity (25°C)	Phytate (mg/g)	Oxalate (mg/100 g)	Alkaloids (mg/g)
<i>Moringa oleifera</i>	1.4678±0.0 ^a	0.8891±0.0 ^a	87.78±0.22 ^a	149.916±3.42 ^a	58.28±4.88 ^a
<i>Parinari curatellifolia</i>	1.4678±0.0 ^a	0.8750±0.0 ^a	65.25± 0.05 ^b	169.92±0.61 ^b	102.65±1.89 ^b
Baobab (<i>Adansonia digitata</i>)	1.4640±0.0 ^a	0.8848±0.0 ^a	86.08± 0.04 ^c	210.08±17.80 ^c	1005±1.00 ^c
Soybean (<i>Glycine max</i>)	1.4695±0.0 ^a	0.8650±0.0 ^a	104.32±0.12 ^d	116.04±6.34 ^d	163.50±3.80 ^d
Pigeon pea (<i>Cajanus cajan</i>)	1.4695±0.0 ^a	0.8652±0.0 ^a	240.47±5.24 ^e	632.56±4.90 ^e	ND
Groundnut (<i>Arachis hypogaeae</i>)	1.4627±0.0 ^a	0.9144±0.0 ^a	44.52± 0.40 ^f	75.41±0.40 ^f	323.60±23.84 ^e

For each parameter, means with same superscript were not significantly different (P>0.05).

A. hypogaeae were higher than the values of 2.5 and 3.3 mg/g (Mbagwu et al., 2011) reported in related studies by other authors.

On the other hand, flavonoids content in extracted crude oil was highest in *P. curatellifolia* (327.00±20.24 mg/g) compared to 79.24±1.55 mg/g for *A. digitata* observed in this study. *M. oleifera* oil has previously been reported to contain total flavonoids content of 18±0.01 mg/g oil (Ogbunugafor et al., 2011).

Conclusions

Results from this study have shown that there are differences in quality parameters in oils extracted from conventional and non-conventional sources. It has also been demonstrated that non-conventional sources of oil like *M. oleifera*, *P. curatellifolia* and *A. digitata* have the potential to become new sources of cheap vegetable oil which can be used by consumers. Results have further shown that oils from non-conventional sources have high oil yield values compared to conventional sources with exception of *C. cajan*. The low iodine and peroxide values of the conventional oils suggest that the oils have longer shelf life and are suitable for human consumption

because of their saturation. It is therefore recommended that opportunities for extracting oils from non-conventional sources should be encouraged.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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