Effect of *Saccharomyces cerevisiae* – Induced Fermentation on the Antioxidant Property of Roselle Calyx Aqueous Extract

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ABSTRACT

This study investigated the effects of *Saccharomyces cerevisiae*-induced fermentation on the antioxidant properties of Roselle calyx aqueous extract and determined the physicochemical changes of the fermented extract. Total phenolics, total flavonoid, ascorbic acid content, total monomeric anthocyanin content and DPPH radical scavenging activity of roselle aqueous extract were investigated before and after fermentation. Roselle calyx aqueous extract was fermented for period of 10 days. During fermentation, the extract was evaluated for pH, alcohol (%), titratable acidity (%) and total soluble solids ($^{\circ}$ Brix). Total soluble solids and pH significantly decreased at the end of the fermentation whereas titratable acidity and alcohol content significantly increased. Fermentation caused significant reduction in total monomeric anthocyanin content from an initial value of 3518 ± 30.8 to 1075 ± 28.2 CGE/100 g dry extract whereas significant increase was observed in total phenolic content from 195.75 ± 76.01 to 455.5 ± 1.41 mgGAE/100 g dry extract and ascorbic acid content from 1392 ± 101 to 2028 ± 108 mg AAE/100 g dry extract and total flavonoids increased from 193.0 ± 74.25 to 291.5 ± 4.95 mgQE/100 g dry extract but it was not significant. There was also a significant increase in 1-1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity of roselle calyx aqueous extract from an initial value of 44.15% to final value of 71.10% after fermentation, leading to an increase in antioxidant activity. Therefore, the quantity of phenolic compounds increased with fermentation process. This study showed that roselle calyx aqueous extract fermented by *Saccharomyces cerevisiae* has a better antioxidant activity.

Keywords: Antioxidants, Fermentation, Roselle Calyx, *Saccharomyces cerevisiae*.

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I. INTRODUCTION

Fermentation is a biotechnological technique in which microorganism-producing enzymes are used to develop new products with modified properties. Research has shown that fermentation of food can cause biochemical changes which in turns helps to improves/ maintains the nutritional, shelf-life and sensory properties of food. The extent of changes that could occur during fermentation of food depends upon the microbial enzymes active in the food and the environmental factors that affects the growth of the organism and its enzymatic activities [1].

Yeasts are unicellular microorganisms that belong to the division of fungi known as the Ascomycota and fungi imperfecti. In thousands of years, yeasts have been known to humans as they have been used to carry out fermentation

process of alcoholic beverage and leavening bread [2]. Fruit juice has been used to produce alcoholic beverage using *Saccharomyces cerevisiae* strains and each strain has its own characteristics and impacts special properties [3]. *Saccharomyces cerevisiae* strains have been reported to be best in ethanol tolerance and production [4].

Hibiscus sabdariffa Linn is a shrub that is commonly known as roselle, which is a member of Malvaceae family. It is cultivated in many areas, including Africa and Central America despite being native to Asia [5]. Roselle is of great economic importance, although it is yet to be fully exploited. The calyces of roselle are used in the preparation of a local refreshing beverage in Nigeria called Zobo. Roselle calyces have been reported to contain dry matter, crude fibre content, crude protein content, ash content and moisture content values were 40.45%, 20.83%, 27.32%, 4.67% and 6.79%,

respectively [6]. Roselle calyx is considered to be good sources of invaluable antioxidants.

Antioxidants are molecules that guard cells against the harmful effects of free radicals, thus balancing oxidative stress [7]. Roselle's antioxidant activity is largely attributed to the phenolic compounds present in roselle plant [8]. Frank *et al* [9] studied the influence of roselle water extract on specific antioxidant status in human body; the report proved that the extract boosts systematic antioxidant strength and also moderates oxidative stress in the body. Research has shown that roselle calyx extract have high antioxidant activity potential and numerous health benefits, surprisingly little attention is channeled on its study. These gaps give an opportunity for investigating the impact of fermentation on the phenolic compounds in roselle calyces. Thus, the objective of this present work is to evaluate the effect of controlled fermentation using *Saccharomyces cerevisiae* on the antioxidant property of roselle calyx aqueous extract as well as determination of physicochemical changes of the fermented extract.

II. MATERIALS AND METHODS

A. Sample Collection

Fresh Roselle calyces (*Hibiscus sabdariffa*) were collected in a clean foil paper from a garden at Barnawa Kaduna, Kaduna State Nigeria. The collected sample was transported to Biotechnology laboratory. The Roselle calyx was identified at herbarium section of the Department of Biological Sciences of Nigerian Defence Academy, Kaduna. The sample was air dried at room temperature.

B. Proximate Composition of Roselle Calyx

Roselle calyx (NDABIOH202101) sample was analyzed proximately for moisture content, crude protein, ash, crude lipid and crude carbohydrate using standard methods [10].

C. Sample Processing

Roselle calyx (NDABIOH202101) extract was prepared by boiling 200 g of dried calyces with 1L of distilled water for 15 minutes using low heat. The boiled roselle juice was filtered by passing it through a sieve to remove the calyces. The physicochemical properties and phytochemical contents of the extract were analyzed and recorded prior to fermentation.

D. Isolation and Identification of Yeast Strain used for Fermentation

For the screening of yeast strain used for the controlled fermentation, fresh palm wine was purchased from rural seller shop in Sabo-Tasha Kaduna, Kaduna state. Standard dilution plate count technique was used to obtain the yeast isolates. Adopting the method of Martini *et al* [11] serial dilution of the fresh palm wine sample was prepared using distilled water as diluents. Pure colonies obtained were transferred onto a Yeast Extract Peptone Glucose (YEPG) agar slants for subsequent identification and stored in the refrigerator at 4 °C. Macroscopic and microscopic identification of *Saccharomyces cerevisiae* was done following Barnett *et al* [12]. Sugar fermentative test, temperature tolerance test, ethanol tolerance test was carried

out using the method of Thais *et al.* [13], after which molecular identification of the isolates was done. The DNA extraction of the isolate was performed using AccuPrep Genomic DNA Extraction Kit protocol (K-3032) following the manufacturer's instructions. PCR was done using universal primers for fungi (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3') while Dye terminator cycle sequencing was done using quick start kit with the primers (NL1 5'-GCATATCAATAATAAGCGGAGGAAAAG-3' and NL4 5'-GGTCCGTGTTTCAAGACGG-3').

E. Fermentation of Roselle Calyx Aqueous Extract

The extract was adjusted by increasing the sugar level to 20.35°Brix and extract was pasteurized at 50 °C for 30 minutes. Sodium metabisulphite 60 mg/l was added to stabilize the extract. Fermentation was conducted in 2 L fermentation vat plugged with cotton wool with cork through which fermenting lock was inserted, the fermentation vat contained 1L of roselle extract inoculated with 1mL of 24 h old broth culture of *Saccharomyces cerevisiae* ($>10^5$ cfu/ml). The fermentation process was carried out for 10 days and temperature was maintained at 30 ± 2 °C.

F. Physicochemical Analysis

Total soluble solid (TSS), pH, titratable acidity, and alcohol content of the extract was determined prior to fermentation and during the fermentation period at an interval of 2 days. Titratable acidity of the extract was determined according to standard method of AOAC [14]. Acidity was determined by titration using 0.1 N NaOH and phenolphthalein as an indicator. The pH was measured using pH meter. Total soluble solid was determined using a hand refractometer. Alcohol content was determined using in difference in the °Brix.

G. Determination of Ascorbic Acid Content

The concentration of Vitamin C content in the roselle extract was analyzed before and after fermentation following Folin-Ciocalteu reagent spectrophotometer method. The sample (20 ml) was pipette into 100 ml volumetric flask and 10% TCA (2 ml) solution was also added, then distilled water was used to make it up to 100 ml. the mixture was shaken for 1minute and allowed to stand for 1minute before it was filtered into a conical flask using whatman filter paper. One millimeter of the sample, 3 ml of distilled water and 0.4ml of Folin reagent were added into the test tube. The mixture was then incubated for 10 minutes at room temperature. Absorbance was read at 760 nm on the spectrophotometer. A standard curve was calibrated using ascorbic acid and the result was expressed in milligram of ascorbic acid per 100 g dry extract [15].

H. Determination of Total Phenolic and Flavonoid Content

Total phenolic content (TPC) of the roselle extract was analyzed with Folin-Ciocalteu reagent using the method of Mgaya *et al.* [15]. Three hundred microliter (300 µl) of the sample (duplicate), 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times) and 1.2 ml of sodium carbonate (7.5% w/v) were added into tubes. The test tubes were incubated in the

dark at room temperature and left for 30 minutes. Absorbance at 765 nm was read using UV spectrophotometer. The standard curve was calibrated using gallic acid and TPC was expressed in gallic acid. Aluminum chloride colorimetric method was used for flavonoid determination [16]. Two hundred and fifty microliters of the sample (250 μ L) were mixed with 1.25 ml distilled water, 75 μ L of 5% NaNO₃ and 150 μ L of 10% AlCl₃ in test tubes. Absorbance at 510 nm was read against a blank. A standard curve was calibrated using quercetin. The concentration of total flavonoids was expressed in mg quercetin/ gram dry extract.

I. Determination of Total Monomeric Anthocyanin

Total monomeric anthocyanin (TMA) of the extract was analyzed prior to and after fermentation. Buffer solutions (pH 1 and pH 5) were prepared in different glass tubes and the sample (200 μ L) was introduced into the tubes and mixed properly. Absorbance was measured at two different wavelengths of 520 nm and 700 nm on the spectrophotometer against a blank (distilled water) [17]. The total monomeric anthocyanins were calculated using the formula:

$$A = (A_{520} - A_{700})_{\text{pH}=1.0} - (A_{520} - A_{700})_{\text{pH}=4.5}$$

$$\text{TMA (mg/L)} = \frac{A \times MW \times F \times 1000}{\epsilon \times l}$$

where

A = Difference of the absorbance (520 nm and 700 nm) of the different pH;

MW = Molecular weight (449.2 gmole⁻¹ for cyanidin-3-O-glucoside);

ϵ = Molar absorptivity (26900 Lmole⁻¹cm⁻¹);

F = dilution factor (total volume/volume of extract);

l = the light path through the quartz cell (1 cm).

The result was expressed as mg equivalents of cyanidin-3-O-glucoside.100 g-1 of dry calyces.

J. Determination of Radical Scavenging Activity

Radical scavenging activity of roselle extract was estimated before and after fermentation using 2,2-diphenyl-1-picryl hydrazyl (DPPH). The sample (0.1ml) was pipette into 3.9 ml DPPH in methanol which was freshly prepared and incubated in the dark for 45 minutes. Absorbance was read at 515 nm with spectrophotometer. DPPH (3.9ml) in 0.1 ml methanol served as the control [18]. Radical scavenging activity was calculated in percentage using the equation below:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (\text{absorbance}_{\text{sample}} / \text{absorbance}_{\text{control}})] \times 100.$$

K. Statistical Analysis

Physicochemical analysis, total phenolic and flavonoids contents, total monomeric anthocyanin content and scavenging activity were expressed as mean $\pm$ SD and analyzed using one-way analysis of variance (ANOVA) using SPSS program at P>0.05.

III. RESULTS

A. Morphological, Physiological and Molecular Identification of Yeast Isolates

The result of this study showed that the colonies are creamy in colour. The microscopic morphology of the yeast isolates examined under light microscope with 100x magnification showed that have ovoid shapes (Fig. 1). The sugar fermentation test on the yeast isolates using six different sugars: glucose, galactose, maltose, sucrose, lactose and raffinose showed that the isolates gave positive reaction for all the sugars except lactose (Table I).

Fig. 1. Microscopic Morphology of Isolate A (*Saccharomyces cerevisiae*).

The effect of temperature (25 $^{\circ}$ C, 30 $^{\circ}$ C, 37 $^{\circ}$ C and 45 $^{\circ}$ C) on the growth pattern of the isolates revealed that isolate A and C showed moderate growth at 45 $^{\circ}$ C while isolate B and D showed low growth, and E showed no growth at 45 $^{\circ}$ C. More so, the effect of ethanol on the growth pattern of the isolates carried out at 10, 13 and 15% ethanol revealed that A and E showed an intensive growth on 15% ethanol while B and C showed moderate growth, and D showed low growth. The result of flocculation capacity of the yeast isolates revealed that A, B and E have flocculation abilities while C and D showed no flocculation ability (Table I).

Isolate A was chosen for molecular study because of its ability to sugar fermentation and high tolerance to ethanol and temperature. Molecular identification showed that genomic DNA extraction from the cultured cell (Isolate A) was successful using PCR technique with universal primer for fungi ITS1 and ITS4 (5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3'). The PCR product gave an estimated size of 500bp (Fig. 2). The sequence obtained for the gene was compared against the non-redundant nucleotide sequence collected at NCBI Genbank using the web interface of NCBI-BLAST. BLASTn analysis of the PCR product indicated that the sequence was *Saccharomyces cerevisiae* M18 strain 26S ribosomal RNA gene, partial sequence and has 88.65 % identity with 99% coverage to *Saccharomyces cerevisiae*, with E-value of 2e-161 and was identified as *Saccharomyces cerevisiae* with ID GU080045.1 (Table II).

TABLE I: MORPHOLOGY AND PHYSIOLOGICAL IDENTIFICATION OF THE YEAST ISOLATES

Isolate	Colour	Shape	Flocculation	Sugar Fermentation					Ethanol (%)			Temperature (°C)			
				Glu	Gal	Mal	Lac	Raf	10%	13%	15%	25	30	37	45
A	Creamy	Ovoid	+	+	+	+	-	+	+++	+++	+++	+++	+++	+++	++
B	Creamy	Ovoid	+	+	+	+	-	+	+++	+++	++	+++	+++	+++	+
C	Creamy	Ovoid	-	+	+	+	-	+	+++	+++	++	+++	+++	+++	++
D	Creamy	Ovoid	-	+	+	+	-	+	++	++	+	+++	++	++	+
E	Creamy	Ovoid	+	+	+	+	-	+	+++	+++	+++	+++	+++	+++	-

Key: Intensive growth (+++), Moderate growth (++), Low growth (+), No growth or response (-), Glu(glucose), Gal(galactose), Mal (maltose), Raf (raffinose), Lac, (lactose), Suc (sucrose).

Fig. 2. Agarose Gel Electrophoresis of the Isolate A (*Saccharomyces cerevisiae*).

TABLE II: SEQUENCING AND BLAST ANALYSIS OF ISOLATE A

Organism	Isolate A
GenBank Relative	<i>Saccharomyces cerevisiae</i>
Accession Number	GU080045.1
Coverage	99%
E-Value	2e-161
Identity	88.65%
Accession Length	594

B. Proximate Composition of Roselle Calyx Extract

Table III shows the proximate composition of roselle calyces on dry weight basis. The result showed that moisture content of roselle calyx was 8.55% while the crude protein content was 6.10%. The crude fat, crude fibre, ash content and carbohydrate content were 3.4%, 9.5%, 9.2% and 63.25%, respectively.

TABLE III: PROXIMATE COMPOSITION OF ROSELLE CALYX EXTRACT

Parameters	Values (%)
Moisture Content	8.55
Crude Protein	6.1
Crude Fat	3.4
Crude Fibre	9.5
Ash Content	9.2
Carbohydrate	63.25

C. Physicochemical Changes During Fermentation

The result in Table IV shows that the pH decreased from an initial value of 3.03 ± 0.04 on day 0 to a final pH of

2.705 ± 0.01 at the end of the fermentation process, but a significant difference ($p < 0.05$) was observed from the day 4 to day 10 of the fermentation process. Total soluble solids significantly decreased during fermentation process from an initial value of 20.35 ± 0.49 °Brix on day 0 to 15.87 ± 0.35 °Brix at the end of fermentation. Alcohol content showed a steady increase in percentage as the fermentation progressed; the increase was from an initial undetectable value at day 0 to a value of $2.69 \pm 0.50\%$ at the end of fermentation. A significant difference ($p < 0.05$) was observed from day 6 to day 10 of fermentation process. Total titratable acidity significantly increased from 0.35 ± 0.02 to $1.15 \pm 0.03\%$ during the fermentation process.

TABLE IV: PHYSICO-CHEMICAL CHANGES DURING FERMENTATION

Day	pH	Alcohol (%)	Titratable Acidity (%)	Total soluble solid (°Brix)
0	3.030 ± 0.04	0.00 ± 0.00	0.35 ± 0.02	20.35 ± 0.49
2	2.940 ± 0.04^a	1.50 ± 0.38^a	0.47 ± 0.01^b	18.40 ± 0.14^b
4	2.865 ± 0.02^b	1.41 ± 0.41^a	0.76 ± 0.01^b	17.96 ± 0.20^b
6	2.825 ± 0.01^b	1.98 ± 0.59^b	0.83 ± 0.01^b	16.97 ± 0.54^b
8	2.750 ± 0.03^b	2.42 ± 0.54^b	1.04 ± 0.01^b	16.23 ± 0.45^b
10	2.705 ± 0.01^b	2.69 ± 0.50^b	1.15 ± 0.03^b	15.87 ± 0.35^b

Values are Mean $\pm$ SD for duplicate: a ($P > 0.05$) and b ($P < 0.05$) as compared to Day 0.

D. Effect of Fermentation on Antioxidant Activity of Roselle Calyx Aqueous Extract

Total phenolic content of the fermented roselle calyx aqueous extract increased from 195.75 ± 76.01 to 455.5 ± 1.41 mgGAE/100 g dry extract and it differed significantly from the unfermented extract. Total flavonoid content increased from 193.0 ± 74.25 to 291.5 ± 4.95 mgQE/100 g dry extract but it did not differ from that of unfermented extract (Table V).

Table V shows that the DPPH radical scavenging activity of the fermented extract increased from 44.1% to 71.10% and a significant difference was observed as compared to the unfermented roselle aqueous extract. The total monomeric anthocyanin content of the fermented extract decreased from an initial value of 3518 ± 30.8 to 1075.0 ± 28.2 CGE/100 g dry extract and also a significant difference was observed as compared to the unfermented roselle aqueous extract. The ascorbic acid content of the fermented extract increased from 1392 ± 101 to 2028 ± 108 mgAAE/100 g dry extract and it differed significantly from the unfermented roselle calyx extract.

TABLE V: EFFECT OF FERMENTATION ON TPC, TFC, TMA, AAC AND DPPH RADICAL SCAVENGING ACTIVITY

Sample (Roselle Aqueous Extract)	TPC (mgGAE/100 g dry extract)	TFC (mgQE/100 g dry extract)	A.S (mgA.S/100 g dry extract)	TMA (mgCy-3-O-GE/100 g dry extract)	DPPH (%)
Unfermented	195.75 ± 76.01	193.0 ± 74.25	1392 ± 101	3518 ± 30.8	44.15 ± 0.07
Fermented	455.5 ± 1.41^b	291.5 ± 4.95^a	2028 ± 108^b	1075.0 ± 28.2^b	71.10 ± 3.82^b

Values are mean $\pm$ SD for duplicate; a ($P > 0.05$), b ($P < 0.05$) as compared to unfermented. TPC (Total phenolic content), TFC (Total flavonoid content), TMA (Total monomeric anthocyanins), AS (Ascorbic acid content), DPPH (1-1-diphenyl-2-picryl hydrazyl) scavenging activity.

IV. DISCUSSION

Earlier reports have confirmed that local beverages are good sources of *Saccharomyces cerevisiae* [19], [20]. Bakare *et al.* [4] documented that *Saccharomyces cerevisiae* isolated from palm wine have high tolerance to ethanol. The result of this study agrees with the work of Kurtzman and Fell [21] who stated that *Saccharomyces cerevisiae* isolated from palm wine have ovoid shape, creamy colour and ability to ferment sugars. Chi and Ameborg [22] reported that ethanol is one of the most important stress conditions that can affect yeast due to its high amount produced during fermentation. This study also revealed that *Saccharomyces cerevisiae* could tolerate ethanol at different concentrations and survive at varied temperature. Similarly, Querol [23] confirmed that wine yeast could adapt and survive under some stressful conditions of fermentation.

In this study, all the yeast isolates fermented all the sugars except lactose. The inability to ferment lactose could be due to lack of lactase enzyme which can breakdown lactose during sugar fermentation. This result is similar to the findings obtained by Umeh *et al.* [2].

Flocculation is an important feature that enables an easy separation of finished product which helps to reduce cost of centrifugation process. The work of Thias *et al.* [13] entitled 'Isolation and Characterization of *Saccharomyces cerevisiae* strains of winery interest', revealed that *Saccharomyces cerevisiae* have flocculation ability, which is similar to the result of this study.

The steady decline in pH during the fermentation process of the roselle extract could be attributed to the uptake of nutrients such as ammonium ions and the release of organic acids into medium, thus increasing the acidity of the extract. The finding of Lanchakon *et al.* [24] affirms that the decrease in total soluble solids during fermentation process could be linked to the consumption of sugars present in the medium by the fermenting yeast for growth and metabolism. Alcohol content showed a steady increase as the fermentation progressed; the progressive increase is due to the conversion of sugars to alcohol by the yeast. The rise in total titratable acidity during the fermentation is due to the decrease in pH of the medium [24].

Phenolic compounds and flavonoids are materials that are spread throughout in plants due to secondary metabolism. Scientific research has revealed that roselle calyces are rich in polyphenols and flavonoids that enhance the nutritive value of roselle as these compounds are correlated to their antioxidant property [25]. Nazarni *et al.* [26] reported that total phenolic content (TPC) and total flavonoid content (TFC) determined using Folin-ciocalteu and Aluminium chloride colorimetric methods respectively, generally increase during fermentation of Tigarum flower, and this finding is in agreement with the results of this study. This finding also agrees with Fahad *et al.* [27], as they adopted similar methods and reported an increase in TPC and TFC after the fermentation of five varieties of grape leaves. More so, Tijana *et al.* [28] adopted similar method and reported an increase in TPC on fermentation of selected cereals and pseudo-cereals using *Saccharomyces cerevisiae*. Wang *et al.* [29] stated that the total phenols present in plant material improve after undergoing the process of fermentation and that

the activity of antioxidant could be linked to the increase in the total phenolic compounds. A report from Verzelloni *et al.* [30] had also emphasized that the capability of antioxidants in wine and vinegar is linked to the total amount of phenols present. During fermentation, phenols bearing high molecular weight are usually broken down into their simpler form [1].

Saccharomyces cerevisiae fermentation significantly enhanced the scavenging activity of roselle calyx aqueous extract from 44.15% to 71.10% in this study. This result is in agreement with the findings obtained by Jirasak [31] who reported that fermentation is an improved technique for procuring greater amount antioxidant in Roselle. The total activity of antioxidants could be a consequence of many antioxidants that are present in the extract [32]. The improved activity of antioxidants in this study could be that *Saccharomyces cerevisiae* caused changes in total phenolic content (TPC) and total flavonoid content (TFC) of the extract during the fermentation reaction. Most fungi are known to possess the ability to produce antioxidants. During biochemical reactions that involve fermentation, fungi are able to release enzymes that can breakdown the glucosidic linkages of some hydroxyl group of phenols that are joined to sugar compounds and this degradation in turn increases the amount of free phenols, thus increasing bioactivity of food material [33], [34].

The total monomeric anthocyanin (TMA) content of the extract which significantly decreased at the end of fermentation, may be attributed to the fact that anthocyanin is sensitive and can be easily broken down in the presence of heat, light, oxygen, enzymes, pH etc. [35]. Ruth *et al.* [36] reported similar decrease in the total and individual anthocyanin concentration by fermentation process of strawberry wine and strawberry vinegar. The decrease could be due to the pH change of the extract during the fermentation process. Anthocyanin retention is largely affected by changes in pH [37]. The amount of ascorbic acid of the extract increased at the end of the fermentation process. This result is similar to the findings of Nwafor and Akpomie [38] who reported a similar increase in ascorbic acid content of Roselle drink upon fermentation by *Saccharomyces cerevisiae*.

V. CONCLUSION

The study revealed that Roselle calyx aqueous extract contain phenols, flavonoids and ascorbic acid, and they exert antioxidant activities in the extract. Also, this study showed that *Saccharomyces cerevisiae*-induced fermentation increased the amount phenols, flavonoids and ascorbic acid in the reaction medium, thus improving its antioxidant activity. It can be concluded that Roselle calyx aqueous extract is a good source of antioxidants and fermentation is a better means of improving the antioxidant status of Roselle extract. This study recommends a laboratory test of the fermented Roselle extract on animals, and the molecular identification of genes responsible for antioxidant activity of Roselle calyx.

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