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Palm sugar (Arenga pinata) photo by Grid.id

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Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Biosafety evaluation of ethanolic extract of *Phyllanthus amarus* leaves on liver and kidney of wistar rats

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Manuscript received: 30 December 2021. Revision accepted: 20 March 2022.

Abstract. *Yakubu OE, Abu MS, Innocent OC, Onuche JI. 2022. Biosafety evaluation of ethanolic extract of* Phyllanthus amarus *leaves on liver and kidney of wistar rats. Asian J Nat Prod Biochem 20: 1-5.* Medicinal plants such as *Phyllanthus amarus* Schumach. & Thonn. have played a significant role in maintaining human health since ancient times. Documented evidence shows that the plant has been used to treat various ailments. Still, its biosafety on vital organs such as the liver and kidney has not been specifically studied. The study was designed to ascertain the biosafety of ethanolic extract of *P. amarus* leaves on the liver and kidney of wistar rats. A total of twenty (20) wistar rats were used, divided into 4 groups of 5 animals each (Group A, B, C, D). Group B, C, D received 100 mg/kg, 200 mg/kg, 400 mg/kg of the extract, respectively. Group A (Control) received only distilled water. The treatment was carried out for two weeks. At the end of the experiments, the animals were sacrificed. Afterward, liver function and renal function evaluation were conducted. The indicators of liver function include Total protein (TP), aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), albumin (ALB), direct bilirubin (DB), and total bilirubin (TB) were determined. The indicators of renal function include sodium ion (Na^+), potassium ion (k^+), chlorine ion (Cl^-), urea, and creatinine. The results showed that the parameters of the liver function of treated animals were no significant ($p>0.0$) difference compared to the normal rats (control). Concentrations of sodium ions (Na+), potassium ions (k+), chlorine ions (Cl-), urea, and creatinine of the treated animals were statistically comparable to the normal rats at ($p<0.05$). This research showed that *P. amarus* did not induce hepatotoxicity or nephrotoxicity at the dose of 100-400 mg/kg BW. Furthermore, this finding has validated the use of *P. amarus* as a traditional herb for treating and managing several diseases, including kidney and liver diseases, without any significant organ damage.

Keywords: Biosafety, ethanolic extract, kidney, liver, *Phyllanthus amarus,* wistar rats

INTRODUCTION

Medicinal plants have continued to play a dominant role in maintaining human health since ancient times. Most modern medicinal drugs are of plant origin (Tuhinadri and Sami 2015) and are essential in drug development programs of the pharmaceutical industry. The knowledge generated over decades has revealed medicinal plants such as *Phyllanthus amarus* Schumach. & Thonn. have been effective in the traditional management of various diseases. Based on these conditions, about 80% of the world population depends solely on traditional (Emeka et al. 2013) or herbal medicine to treat various diseases, especially in Africa and other developing nations (Ilonga et al. 2018; Yakubu et al. 2020a). However, the historical role of medicinal herbs in treating and preventing disease does not assure their safety for uncontrolled usage by an uninformed public (Matthews et al. 1999). Most medicinal plants have relatively non-toxic potential or adverse effects when used by humans; however, some medicinal plants have a high level of toxicity. They have the possibility of damaging organs. For this reason, caution in the use of medicinal plants is presently on the increase due to easy availability, affordability, accessibility, and promising efficacy compared to the often-high cost and adverse effects of the standard synthetic drug (Yakubu et al. 2020c).

Phyllanthus amarus is a broad-spectrum medicinal plant that has received worldwide recognition (Srividiya and Perival 1995). They are widely distributed in most tropical and subtropical countries and have long been used in traditional medicine to treat chronic liver diseases (Liu et al. 2003). *P. amarus* is generally employed to reduce pain, expel intestinal gas, stimulate and promote digestion, as anti-helminths to expel intestinal worms, and act as a mild laxative. *P. amarus* also has antiseptic, diuretic, antiviral, antidiabetic, hypotensive, antipyretic, and it is also used to treat jaundice, diarrhea, dysentery, wound ulcers, and urogenital diseases (Calixto et al. 1998). The plant extracts have been found to contain high levels of saponins, tannins, flavonoids, and alkaloids (Fernand 1998; Naaz et al. 2007; Krithika and Verma 2009). *P. amarus* has been classified as having a low potential for toxicity, with an LD_{50} averaging 2000 mg/kg/day (Krithika and Verma 2009). However, the toxicity of this plant on vital organs such as the liver and kidney needs to be revalidated, considering its increased demand for several herbal medicine preparations. Therefore, this study was designed to ascertain the biosafety of ethanolic extract of *P. amarus* leaves on the liver and kidney of wistar rats.

MATERIALS AND METHODS

Collection of plant

The fresh leaves of *P. amarus* were collected in December 2019 within the Biological garden of Federal University Wukari Taraba State, Nigeria. The plant was identified and authenticated in the herbarium of the Biological Science Department, Federal University Wukari, Nigeria. The fresh leaves were air-dried for three (3) days and pounded into a fine powder using mortar and pestle.

Extraction

The dried material (500 g) was macerated in 2000 mL of ethanol (1:4 w/v) for 48 hours at room temperature, according to Yakubu et al. (2014). It was continually stirred every 5 hours. After finishing maceration, the filtrate was sieved with clean white mesh before filtering using Whitman No 1 filter paper. Next, the filtrate was poured into a beaker and air-dried. Finally, the resultant extract (18 g) was placed into the incubator for further studies.

Animals management

Twenty healthy adult wistar rats of both sexes were obtained and maintained in the animal house of Biochemistry Department, Faculty of Pure and Applied Science, Federal University Wukari, Taraba State, Nigeria. They were acclimatized for two weeks and fed with growers mash and water ad libitum.

Experimental design

The wistar rats of the average weight of 177 g were randomly assigned into four (4) groups: A, B, C, and D of five (5) animals in each group. Group B, C, D served as treatment groups, while group A served as the control. The rats in the treatment groups (B, C, and D) received 100 mg/kg, 200 mg/kg, and 400 mg/kg body weight of ethanolic extracts of *P. amarus* orally through an or gastric tube daily, respectively. The control group received an equal volume of distilled water without the *P. amarus* extract. After the experimental period, animals were sacrificed under chloroform anesthesia, venous blood was collected by cardiac puncture, and liver was harvested. Blood samples were collected, and serum was obtained by centrifuging at 3000 rpm for 5 min.

Group A: Normal control received distilled water
Group B: 100 mg/kg/p.o/day of extract
Group C: 200 mg/kg/p.o/day of extract
Group D: 400 mg/kg/p.o/day of extract

Determination of biochemical parameters

Analysis of Aspartate Aminotransferase (AST) activity

AST activity was determined by the method described by Amador and Wacker (1962).

Principle: L-Aspartate and α-Ketoglutarate react in the presence of AST in the sample to yield oxaloacetate and L-glutamate. The oxaloacetate is reduced by malate dehydrogenase to yield L-malate with NADH oxidation to NAD^+. The reaction is monitored by measuring the decrease in NADH absorbance at 340 nm. The reduction rate in absorbance is proportional to AST activity in the sample.

Procedure: To 1 mL of reagent added to all required test tubes, 0.05 mL of the sample was added to the sample test tube and none to the blank. It was incubated at room temperature for 20 minutes, mixed immediately, and the first absorbance of the test was read exactly at 1 minute and after that at 30, 60, 90, and 120 seconds at 340 nm. The mean change in absorbance per minute was determined, and the test results were calculated as follows :

Serum AST activity (IU/L) = Change in A/min × 3376.

Assessment of Alanine Aminotransferase (ALT) activity

ALT activity was determined by the method described by Amador and Wacker (1962).

Principle: L-alanine and α-ketoglutarate react in the presence of ALT in the sample to yield Pyruvate and L-glutamate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with oxidation of NADH to NAD. The reaction is monitored by measuring the decrease in absorbance at 340 nm. The rate of reduction is proportional to ALT activity in the sample.

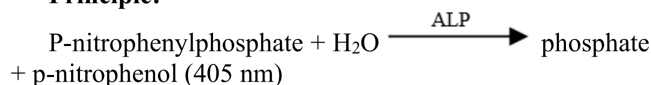
Procedure: To 1 mL of reagent added to all required test tubes, 0.05 mL of the sample was added to the test sample tube test and none to the blank. It was incubated at room temperature for 20 min, it was mixed immediately, and the first absorbance was read at precisely 1 minute and after that at 30, 60, 90, and 120 seconds at 340 nm. The mean change in absorbance per minute was determined, and test results were calculated as follows:

Serum ALT activity (IU/L) = Δ**A**/min × 3376.

Assessment of Alkaline Phosphatase (ALP) activity

The serum activity of Alkaline Phosphatase (ALP) was determined by the method Haussament (1977) described.

Principle:



P-nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ phosphate + p-nitrophenol (405 nm)

Alkaline Phosphatase in a sample hydrolyses para nitrophenyl phosphate into para nitrophenol and phosphate in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm and 37^0C due to liberation of para nitrophenol is proportional to the alkaline phosphatase activity.

Procedure: Reagent (1 mL) containing diethanolamine buffer, magnesium chloride, and substrate (P-nitrophenyl phosphate) was added into a clean test tube and incubated at 37^0C followed by the addition of 0.02 mL of sample. The mixture was mixed thoroughly, and immediately absorbance of the sample was read precisely at 30, 60, 90, and 120 seconds at 405 nm against the reference blank (distilled water). The mean change in absorbance per minute was determined, and the test results were calculated.

The ALP activity was calculated using the following formulae:

Serum alkaline phosphatase activity (IU/L) = Δ**A**/min x 2713.

Serum creatinine and urea

Serum creatinine and urea were determined using auto-analyzer: Selectra ProM, while the determination of Serum Electrolytes was carried out using the Ion-Selective Electrode (ISE) method (Burnett et al. 2000)

Statistical analysis

The mean ± SD of all values was calculated, and change observed between the treatment groups and control was subjected to analysis of variance (ANOVA) using SPSS version 23. Differences between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Effect of *Phyllanthus amarus* leaves extract on serum electrolytes of wistar rat

The effect of the *P. amarus* leaves extract on some serum electrolytes levels is depicted in Table 1. There was no significant ($p<0.05$) difference between the rats administered with extract and the normal/control rats. However, there was a significant ($p<0.05$) decrease in the Na^+ level of animals that received 100 and 200 mg/kg of the extract compared to the control animals. Similarly, a significant ($p<0.05$) reduction was observed in the K^+ level of the group that received 100 mg/kg compared to the other extract groups, including the control group.

Effect of *Phyllanthus amarus* leaves extract on urea and creatinine level in wistar rat

It was observed that all the groups, including the extract administered and the control groups, showed no significant ($p>0.05$) differences in the levels of urea and creatinine, as represented in Table 2.

Effect of *Phyllanthus amarus* leaves extraction liver enzyme of wistar rat

Table 3 showed that the liver enzymes' activity was significantly ($p<0.05$) high or low in the group that received 200 mg/kg compared to the control group. However, the other extract administered groups showed no consistent difference in the enzymes' activity compared with the control.

Effect of *Phyllanthus amarus* leaves extract on some liver function biomarkers of wistar rat

Rats treated with the extract at different doses did not show significant ($p>0.05$) difference in the total protein levels, total bilirubin, direct bilirubin, and albumin, as represented in Table 4 when compared to the control rats.

Table 1. Effect of *Phyllanthus amarus* leaves extract on serum electrolytes of wistar rat

Treatment	Na^+(mmol/L)	K^+ (mmol/L)	CL^-(mmol/L)
Control	$238.6±1\ 34^b$	$38.7±5.55^b$	$145.0±25.14^a$
Extract 100 mg	$171.0±06.74^a$	$36.18±7.29^b$	$136.4±19.06^a$
Extract 200 mg	$185.6±05.85^a$	$19.5±5.99^a$	$145.6±19.96^a$
Extract 400 mg	$223.6±26.15^b$	$39.7±6.08^b$	$149.4±19.93^a$

Note: Results represent mean ± standard deviation of group results obtained (n=5). Values having different superscripts along the column are considered statistically significant ($p<0.05$)

Table 2. Effect of *Phyllanthus amarus* leaves extract on urea and creatinine level in wistar rat

Treatment	Urea (mmol/L)	Creatinine (mmol/L)
Control	$10.44±5.01^a$	$5.12±3.21^a$
Extract 100 mg	$0.7.84±1.65^a$	$4.96±3.68^a$
Extract 200 mg	13.52±5.67a	$3.12±3.64^a$
Extract 400 mg	$12.68±9.29^a$	$1.92±2.10^a$

Note: Results represent mean ± standard deviation of group results obtained (n=5). Values having different superscripts along the column are considered statistically significant ($p<0.05$)

Table 3. Effect of *Phyllanthus amarus* leaves extraction liver enzyme of wistar rat

Treatment	ALT (IU/mL)	AST (IU/mL)	ALP (IU/L)
Control	$3.8±1.30^a$	$5.4±0.54^a$	$16.0±4.06^c$
Extract 100 mg	$11.4±1.34^b$	$5.54±3.13^a$	$13.2±5.11^{bc}$
Extract 200 mg	$20.4±6.46^c$	$10.4±2.07^b$	$7.8±1.30^a$
Extract 400 mg	$2.54±0.95^a$	$4.34±1.74^a$	$9.2±3.42^b$

Note: Results represent mean ± standard deviation of group results obtained (n=5). Values having different superscripts along the column are considered statistically significant ($p<0.05$)

Table 4. Effect of *Phyllanthus amarus* leaves extraction on some liver function biomarkers of wistar rat

Treatment	ALB (mg/mL)	TP (mg/mL)	DB (µmol/L)	TB (µmol/L)
Control	2.6±1.25[a]	8.98±3.07[a]	30.03±6.34[ab]	30.96±7.70[a]
Extract 100 mg	2.78±0.38[a]	8.74±1.21[a]	30.36±4.58[ab]	34.66±9.89[a]
Extract 200 mg	3.2±0.72[a]	9.62±2.82[a]	33.56±9.11[b]	36.84±6.50[a]
Extract 400 mg	3.42±0.61[a]	11.54±5.82[a]	23.84±4.33[a]	56.86±17.63[a]

Note: Results represent mean ± standard deviation of group results obtained (n=5). Values having different superscripts along the column are considered statistically significant ($p<0.05$)

Discussion

Kidneys and the liver are vital organs that function for homeostasis, regulating electrolytes balance in the blood, excreting waste products of metabolism, secretion of some enzymes and hormones, metabolism, osmoregulation, and detoxification. However, damage and toxic substances in these essential organs can lead to inefficiency in carrying out their functions.

Electrolytes (sodium, potassium, chloride, and bicarbonate) balance in the blood is a good indicator of kidneys and heart functions. Therefore, adequate information on serum electrolytes can assist in the determination of organ functions. For example, liver function assessment can be done by evaluating liver enzyme (AST, ALT, and ALP) parameters, total protein, albumin, and total bilirubin (Yakubu et al. 2021a).

The result revealed a significant decrease ($p>0.05$) in sodium ion (Na^+) concentration between groups administered with doses of extract (100 mg, 200 mg) compared to normal/ control. Still, it showed no significant ($p>0.05$) difference at 400 mg dosage compared to the normal/ control. Therefore, the results suggested mild to no change of sodium ion concentration in the serum of rats administered with *P. amarus*. Furthermore, the result also revealed no significant ($p>0.05$) change in the concentration level of the other electrolytes, i.e., chlorine (Cl^-) and Potassium (K^+) between animal groups administered with the extract and normal control. These findings imply that the administration of *P. amarus* extract within these experimental doses did not significantly change the electrolytes concentrations in the serum of the wistar rats. This result agrees with the work of Krithika and Verma (2009) that the LD_{50} value of *P. amarus* was high or categorized as non-toxic. The result also revealed no significant difference in serum urea and creatinine concentration between and within the groups of the administered dose of 100 mg, 200 mg, and 400 mg compared to normal control. Hence, the data from the electrolytes values, urea, and creatinine in the serum indicates that the extract did not exact any deleterious effect on the kidney or negatively affect the heart.

The liver is an essential organ that carries out the biological system's metabolic, secretion, storage, and detoxification functions. Therefore, direct or indirect damage to the hepatocytes will cause alteration of these functions, thereby affecting the organism's living condition (Yakubu et al. 2020b). The liver parameters results showed no significant ($p>0.05$) difference in the level of ALB, TP, TB, DB, ALT, AST, and ALP of the animal groups administered with extract compared to the normal control. Based on the parameters observed, it shows that the administration of the extract did not cause damage or toxicity to the liver. *P. amarus* contains alkaloids and a high concentration of antioxidant compounds (Fernand 1998; Naaz et al. 2007; Krithika and Verma 2009), essential for reducing oxidative stress. It may also have been the major contributing factor for stabilizing all the hepatic parameters (Yakubu et al. 2021b). However, inappropriate use of antioxidants can induce oxidative stress (Galati and O'Brien 2004; Atici et al. 2005); hence the use of *P. amarus* extract should be kept at an optimal level.

It can be concluded that administration of *P. amarus* extract up to 400 mg/kg BW did not induce hepatotoxicity or nephrotoxicity. Furthermore, this finding has validated the use of *P. amarus* as a traditional herb for treating and managing several diseases, including kidney and liver diseases, without any significant organ damage.

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Comparison of phytochemical constituents of ethanol leaf extracts of *Solanum macrocarpon* and *Vernonia amygdalina*

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Abstract. *Oyesola OA, Sampson II, Augustine AA, Adejoke OB, Taiwo GE. 2022. Comparison of phytochemical constituents of ethanol leaf extracts of* Solanum macrocarpon *and* Vernonia amygdalina. *Asian J Nat Prod Biochem 20: 6-10.* A plant can exert physiological changes on biological systems due to phytochemicals present in plants parts. This study evaluated and compared the phytochemical constituent (both quantitative and qualitative) and the antioxidant properties of the ethanol leaf extract *Solanum macrocarpon* L.and *Vernonia amygdalina* Delile using standard methods. Tannin, phenol, cardiac glycoside, alkaloid, and flavonoid were present in both plant extracts. Alkaloid, flavonoid, and cardiac glycoside in large quantities in *S. macrocarpon* compared to *V. amygdalina*. At the same time, *V. amygdalina* contained a large amount of tannin and phenol. Reducing sugar was present in *S. macrocarpon and* absent in *V. amygdalina*, saponin and steroid were present in *V. amygdalina* and absent in *S. marcrocarpon*, terpenoid and phlobatanin were both missing in the leaf extract of both plants. In contrast, both plant extracts showed radical scavenging activities, with *V. amygdalina* having a higher antioxidant capacity than *S. macrocarpon.* The present study's findings indicated that the ethanol leaf extract of *S. macrocarpon* and *V. amygdalina* possess antioxidant properties and may be effective against oxidative stress.

Keywords: Antioxidant, phytochemical, *S. macrocarpon, V. amygdalina*

INTRODUCTION

Plants' medicinal properties are attributed to synthetic components that have a distinct physiological effect on the human body; these compound substances are known as phytochemicals (Edeoga et al. 2005). Phytochemicals are currently receiving more attention because of their effectiveness in treating infectious diseases (Wamuyu et al., 2020). The phytochemical analysis of medicinal plants involves extracting, screening, and identifying bioactive compounds in different plant parts. Flavonoids, alkaloids, carotenoids, tannins, antioxidants, and phenolic compounds are some bioactive molecules obtained from plants. Phytochemicals are naturally found in plants and play an essential role in assisting plants to protect themselves against pathogenic microbes by demonstrating antimicrobial activity through hindrance or killing mechanisms. The discharge of these mixtures varies from one plant to the next, with some producing higher quality and others producing low quality (Tariq and Reyaz 2013). Research has found that phytochemicals present in fruits and vegetables decrease the risk of cancer, act as antioxidants, and may treat or manage infections and metabolic disorders (Abbasi et al. 2015). *Solanum macrocarpon* L. is the scientific name for the African eggplant, which belongs to the Solanaceae family and the plant genus Solanum (Agoreyo et al. 2012). Eggplants have a wide range of nutritional and therapeutic properties, making them a beneficial complement to any diet because they contain a significant amount of nutrients and phytochemical compounds such as saponins, phenols, flavonoids, and tannins, among others (Ibiam and Nwigwe 2013). Eggplant fruit aids in the prevention, management, and treatment of various diseases by lowering blood cholesterol levels, managing high blood pressure, reducing weight, and having anti-haemorrhoidal and anti-glaucoma properties (Ossamulu et al. 2014).

Because of its bitter taste, *Vernonia amygdalina* Delile is also called a bitter leaf. A small evergreen shrub grows throughout Africa and belongs to the Asteraceae family. It was reported to be a plant that can help with diabetes and fever management (Imaga and Bamigbetan 2013). The bitter taste of *V. amygdalina* is due to the presence of sesquiterpene lactones (vernodalin, vernolepin, and vernomygdin) and steroid glycosides (vernonisides) (Ojimelukwe and Amaechi 2019). Previous phytochemical studies on the leaf extract of *V. amygdalina* shows the presence of bioactive compounds like tannins, saponins, flavonoid, glycosides, alkaloids, and steroid. Traditionally, plants are also used to treat and manage malaria, intestinal parasite, diarrhea, and high blood sugar (Udochukwu et al., 2015). African eggplant and bitter leaf are important vegetables in African communities due to their nutritional and medicinal value; the study will compare and analyze the phytochemical constituent and in-vitro antioxidant properties of the ethanol leaf extract of *S. macrocarpon* and *V. amygdalina*; it will also add to the knowledge on their significance use in ethnomedicine.

MATERIALS AND METHODS

Plants material

Matured leaves of bitter leaf plants were collected from a family garden in the Sagamu local government area of South-West Nigeria. In contrast, matured leaves of African eggplant were bought from a local market in the Sagamu local government area of South-West Nigeria. In addition, authentications of the leaves sample were carried out at the department of plant science, faculty of science, Olabisi Onabanjo University, Nigeria.

Preparation of the ethanol leaves extract of African eggplant and bitter leaf plant

The leaves of African eggplant and bitter leaf plant were air-dried and powdered using a blender; 150 g of the blended leaves were soaked in 750 mL of ethanol (70% ethanol and 30% water) for three days and then filtered. After filtration, the filtrate was heated at a temperature of 40°C for evaporation.

Determination of percentage yield of African eggplant and bitter leaf plant

The percentage of African eggplant extract was determined by calculating the percentage of the weight of the extract to the original weight before drying the sample, using the formula;

$$percentage\ yield = \frac{weight\ of\ extract}{weight\ of\ sample} \times \frac{100}{1}$$

Weight of African eggplant= 150g
Weight of dried shaft of African eggplant= 73.4g
Weight of extract = 150g — 73.4g= 76.6g

P$$ercentage\ yield = \frac{76.6g}{150g} \times \frac{100}{1} = 51.0\%$$

The percentage yield for African eggplant is 51.0%

The percentage yield for bitter leaf plant was also calculated using the same formula stated above;

$$percentage\ yield = \frac{weight\ of\ extract}{weight\ of\ sample} \times \frac{100}{1}$$

Weight of bitter leaf plant – 150g
Weight of dried shaft of bitter leaf plant- 70.10g
Weight of extract = 150g—70.10g= 79.9g

$$percentage\ yield = \frac{79.9g}{150g} \times \frac{100}{1} = 53.3\%$$

The percentage yield for bitter leaf is 53.3g

Phytochemical screening and in-vitro antioxidant procedure

Phytochemical tests were carried out on the ethanol leaf extract of *V. amygdalina* and *S.macrocarpon* using the standard procedure to identify the constituents present and in-vitro antioxidant enzymes activity as described by Harborne (1973), Trease and Evans (1989), Sofowra (1993), and Alisi and Onyeze (2008).

RESULTS AND DISCUSSION

Determination of the phytochemical present in the ethanol extract of the leaves extract of *Solanum macrocarpon* and *Vernonia amygdalina*

Tables 1 and 2 show the qualitative and quantitative (mg/100 g) analysis of the phytochemical constituents in the ethanol leaf extract of *S.macrocarpon* and *V. amygdalina*. The results revealed the presence of bioactive compounds in the extract studied from the table; the results show that phenols and tannins, flavonoids, and cardiac glycoside were present in both plant extracts. However, saponins were absent only in the ethanol extract of the African eggplant, and reducing sugar was absent in the ethanol extract of the bitter leaf plant. At the same time, steroids were missing in the ethanol extract of African eggplant. In addition, terpenoid and philobatanin were not present in both plant extracts. The results also show that the leaves of bitter leaf contained high levels of tannin and phenol compared to the leaves of African eggplant. In contrast, the leaves of African eggplant contain more alkaloids, cardiac glycoside, and flavonoids compared with the leaves of bitter leaf.

Plant phytochemical constituents are increasingly linked to the elicited physiological activities; in traditional medicine, plant parts are used to manage and treat various disorders (Gurib-Fakim 2006). The phytochemical screening results in Table 3 show the presence of active entities that elicit significant pharmacological and physiological responses. The presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, terpenoids, reducing sugar, steroid, and cardiac glycosides was observed. Tannins and flavonoids in the plant extract are responsible for the observed DPPH radical scavenging activity, as seen in Table 3. Flavonoids and tannins are phenolic compounds, which are the most abundant bioactive compounds in plants that act as antioxidants or free radical scavengers. The presence of one bioactive compound in the plant extract and its absence in another may be due to a difference in solvent polarity, which follows the rules of thumb" like dissolves like" (Adamu et al. 2019). The bitterness of African eggplants and bitter leaf plants is caused by the presence of alkaloids, primarily glycoalkaloids, and the degree of bitterness determines the edibility or otherwise. In general, ethanol extracts of bitter leaf plants contained more phytochemicals than ethanol extracts of African eggplant. The saponins found in the samples are major nutritional substances and nutraceuticals. Previous research studies have shown that medicinal plants' saponins reduce glycoside toxicity by hydrolyzing terpenoids (Xu et al. 1996; Chinedu et al. 2011). The antioxidant activity shown by the plant extracts in Tables 3-6 may result from the presence of phenolic compounds. The antioxidant action of phenolic compounds stems from their redox characteristics, which can aid the

absorption and neutralization of free radicals, singlet and triplet oxygen quenching, and the decomposition of peroxides (Al-Shaya et al. 2020). The food industry is increasingly interested in crude extracts of high phenolic medicinal plant materials (Osawa 1994).

Alkaloids are essential for plant protection and survival because they protect them from microorganisms' activities, insects and herbivores, and other plants (allelopathically active chemicals) (Molyneux et al. 1996). Plants containing alkaloids have been used as dyes, spices, drugs, and poisons almost since the beginning of human history. Cardiac glycosides are derived from steroids, and they act primarily on the cardiac muscle; they are potent in managing heart disease. Congestive heart failure causes an influx of Na^+ and an outflow of K^+ during each heart contraction. Na^+, K^+-ATPase must re-establish the concentration gradient before the next contraction by pumping Na+ into the cell against a concentration gradient. Cardiac glycosides inhibit Na^+ K^+-ATPase, increasing and increasing the force of myocardial contraction as a result (Farnsworth 1966); cardiac glycosides also have antitumor activity (Doskotch et al. 1972). Other studies have also reported the presence of a physiologically active substance in bitter leaf (Usunobun and Okolie 2015; Usunomena and Ngozi 2016) and African eggplant (Ilodibia et al. 2016; Eletta et al. 2017).

Table 1. Quantitative determination of phytochemical constituents of ethanol leaves extract African eggplant and bitter leaf plant

Sample	Tannin mg/100 g	Alkaloid mg/100 g	Reducing sugar mg/100 g	Cardiac glycoside mg/100 g	Phenol mg/100 g	Steroid mg/100 g	Flavanoid mg/100 g	Saponin mg/100 g
V. amygdalina	45.09	39.14	-	38.11	61.93	-	51.93	51.99
S. macrocarpon	39.80	69.61	45.90	38.30	41.21	31.25	67.51	-

Table 2. Qualitative determination of phytochemical constituents of ethanol leaves extracts African eggplant and bitter leaf plant

Sample	Tannin	Alkaloid	Reducing sugar	Cardiac glycoside	Terpenoid	Phenol	Phlobatanin	Steroid	Flavanoid	Saponin
V. amygdalina	+	+	-	+	-	+	-	+	+	+
S. macrocarpon	+	+	+	+	-	+	-	-	+	-

Note: + =present, - =absent

Table 3. DPPH radical scavenging activity of ethanol leaf extracts of *Solanum macrocarpon* and *Vernonia amygdalina*

Sample	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
V. amygdalina	31.25	47.06	59.44	77.72
S. macrocarpon	33.65	50.22	56.90	63.50

Table 4. Nitric oxide scavenging activity of ethanol leaf extracts *Solanum macrocarpon* and *Vernonia amygdalina*

Sample	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
V. amygdalina	37.88	48.07	61.29	77.27
S. macrocarpon	38.22	41.89	53.06	61.66

Table 5. Reducing power of ethanol leaf extracts *Solanum macrocarpon* and *Vernonia amygdalina*

Sample	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
V. amygdalina	0.12	0.26	0.32	0.46
S. macrocarpon	0.17	0.21	0.36	0.48

Table 6. Total flavonoid, total phenol, and total antioxidant activity of ethanol leaf extract *Solanum macrocarpon* and *Vernonia amygdalina*

Sample	Total flavonoid mg/100 g	Total phenol Mg/100 g	Total antioxidant capacity
V. amygdalina	51.92	61.93	52.07
S. macrocarpon	67.51	27.45	39.71

Evaluation of the antioxidant activity of ethanol leaves extract of *Solanum macrocarpon* and *Vernonia amygdalina*

Total Antioxidant Capability (TAC) was coined to describe the antioxidant's reducing capacity in a single metric. TAC of the ethanol extracts of was *S. macrocarpon* and *V. amygdalina* calculated using a variety of methodologies, including a DPPH radical scavenging activity assay and a nitric oxide scavenging activity, and a reducing power assay. In the DPPH radical scavenging activity assay, nitric oxide scavenging activity assay, and reducing power assay, absorbance directly represents reducing power (McCord 2000). Tables 3-6 show the ethanol extract's antioxidant activities of bitter leaf and African eggplant. The results of the DPPH scavenging activity of the extracts are shown in Table 4. The African eggplant and bitter leaf plant ethanol extract exhibited concentration-dependent antiradical activity by inhibiting DPPH radical with inhibitory concentrations of 63.50 at 100g/ml and 77.72 at 100 g/mL, respectively. researches commonly utilize the model system of DPPH radicals to explore the scavenging ability of various medicinal plant products (Benslama and Harrar 2016). Due to the hydrogen donating ability of antioxidants, they can scavenge DPPH radicals (Baumann 1979). Free radicals scavenger are important to prevent the harmful effects of free radicals. The DPPH free radical scavenging methods are widely used to evaluate the antioxidant properties of plants extracts, the DPPH radical scavenging activity of *V. amygdalina* corresponds with the study of Erasto et al. (2007), Ho et al. (2012), Atangwho et al. (2013), while that of *S. macrocarpon* correspond with the study of Adewale et al. (2014) and Eletta et al. (2017). In the reducing power assay (Table 5), the presence of antioxidants in the samples would result in the extract donating an electron to reduce Fe^{3+} to Fe^{2+}. The extracts with reducing power reveal that they are electron donors, reduce oxidized intermediates, and act as primary antioxidants (Chanda and Dave 2009). The reducing power is frequently employed to access the antioxidant activity of natural plant products. The presence of reductants which act as an antioxidant by breaking free radical chains by donating a hydrogen atom, is often associated with the existence of reducing power (Rahman et al. 2015). Observation from Table 5 shows that the reducing power of both plant extracts was in different concentrations. These results also correspond with other studies which show that *S. macrocarpon* (Adewale et al. 2014; Famuwagun et al. 2017) and *V. amygdalina* (Ho et al. 2012; Adesanoye and Farombi 2014) have reduced power activity, but the ethanol leaf extract of *S. macrocarpon* had more reducing power when compared to the ethanol leaf extract of *V. amygdalina*. Nitric oxide (NO) or reactive nitrogen species such as NO_2, N_2O_4, N_3O_4, NO_3, and NO2 are formed during reactive nitrogen reactions with oxygen or superoxides. These compounds alter many cellular components' structural and functional behavior. Plant products can inhibit the detrimental consequences of excessive NO production in the human body, which could be of considerable interest prevent the harmful effects of excessive NO production. NO has also been linked to inflammation, cancer, and other diseases (Moncada and Higgs 1993). Table 5 shows the relative NO scavenging potential of African eggplant and bitter leaf plant ethanol extracts. Since its identification as a new signal molecule, NO is linked to a range of physiological reactions. It produces vascular dilation by transmitting signals from vascular endothelial cells to vascular smooth muscle cells. It also plays an important part in respiratory, immunological, neuromuscular, and other physiological activities (Ebrahimzadeh et al. 2010). Other studies reported the NO scavenging activity of *S. macrocarpon* and *V. amygdalina* (Ng et al. 2015; Omede et al. 2018). Phenols are secondary metabolites found in plants. The majority of the plant products can cause a pharmacological effect which includes; anti-inflammatory, antispasmodic, and anti-allergic, among others. Most pathological diseases and infections such as diabetes, cancer, and cardiovascular disease resulting from the oxidative injury are caused by oxidative stress. The accumulation of reactive oxygen species (ROS) triggers a series of reactions that breaks down organic molecule such as DNA, lipids, and protein in the body, which are the main causes of disease (Halliwell et al. 1992; Craig 1999; Exarchou et al. 2002; Afanas'ev 2010). Many plant extracts that contain phenol compounds possess antimicrobial and antioxidant properties and are used to treat and manage disease (Choi et al. 2010). In Table 6, it was observed that the ethanol leaf extract of *V. amygdalina* has a high quantity of phenols compared with the ethanol leaf extract of *S. macrocarpon*. At the same time, total flavonoid content was high in the ethanol leaf extract of *S. macrocarpon* compared with *V. amygdalina*. The antioxidant activities of these plant extracts are due to the presence of flavonoid and phenol compounds.

In conclusion, the presence of tannins, alkaloids, cardiac glycoside, phenol, and flavanoid in the ethanol leaf extracts of *V. amygdalina* and *S. macrocarpon* are responsible for the physiological changes exerted by the plant on biological systems and also possess antioxidant properties and may be effective against oxidative stress caused by free radicals. However, more studies are needed to isolate the active ingredients present in the combination of African eggplant and bitter leaf ethanol extracts and study their anti-inflammatory effect.

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Effect of palm sap (*Arenga pinnata*) on blood glucose levels of mice (*Mus musculus*) alloxan-induced diabetic

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Abstract. *Liputo SM, Lamondo D, Solang M. 2021. Effect of palm sap (*Arenga pinnata*) on blood glucose levels of mice (*Mus musculus*) alloxan-induced diabetic. Asian J Nat Prod Biochem 20: 11-15.* This study aims to determine the effect of palm sap (*Arenga pinnata* (Wurmb) Merr.) in reducing blood glucose levels in alloxan-induced diabetic mice (*Mus musculus* Linnaeus, 1758). Twenty-five mice aged 2-3 months were grouped into a negative control group, a positive control group and a palm sap group (0.2 mL, 0.4 mL, and 0.6 mL). The result showed that the palm sap with a dose of 0.4 mL was the most significant in reducing blood glucose levels. This study concluded that the administration of palm sap affected decreasing blood glucose levels ($p < 0.05$).

Keywords: Alloxan, blood glucose, mice, palm sap

INTRODUCTION

Diabetes is one of the fastest-growing health emergencies compared to other diseases. Diabetes mellitus is a condition in metabolism defect that features hyperglycemia symptoms, which require constant medical care. DM can cause many acute and seriously chronic complications, such as diabetic ketoacidosis, nonketotic hyperosmolar coma, cardiovascular disease, chronic kidney failure, foot ulcers and damage to the eyes. Based on data from the International Diabetes Federation (IDF), in 2019, people with diabetes reached 465 million adults in the world and it is predicted that in 2045 700 million adults will suffer from this disease or an increase of 51% in 2045 (IDF 2019).

As a developing country, Indonesia has experienced an increase in diabetes mellitus cases. Diabetes affected 7.7 million adults (20-79 years old) in 2013 and 10.7 million adults in 2019. (IDF 2019). According to National Health Survey(Riskesdas) (2018), the number of DM sufferers aged 15 years has increased by 2% compared to 2013. Gorontalo is one of the provinces in the country with the highest number of people with diabetes. Based on Riskesdas (2018), the prevalence of diabetes mellitus in Gorontalo has increased to 2.4 percent which only 1.5% in 2013.

Synthetic chemicals and traditional medicines are effective treatments for DM (Diabetes Mellitus) patients. One of the synthetic drug classes that are most often used to treat DM is glibenclamide because it is widely marketed and the price is affordable. However, glibenclamide has side effects, such as nausea, constipation, dizziness, tremors, and hypoglycemia (Putra et al. 2017). Therefore, as stated by WHO (2004), traditional medicine is used as self-medication or alternative treatment, particularly for chronic diseases such as diabetes and endocrine disorders. Additionally, although WHO recommends traditional medicine, scientific research is still needed to determine the truth of its efficacy, for example, palm sap (*Arenga pinnata* (Wurmb) Merr.).

Aren is one type of palm plant that is almost spread throughout Indonesia. Almost all parts of this plant can be utilized and used for various needs, both from parts of plant organs (roots, stems, leaves, fruit, etc.), as well as their production (sap, starch/flour) (Lempang 2012; Gunawan et al. 2017). According to Kandowangko et al. (2011), palm sap (*A. pinnata*) has long been utilized as an antidiabetic medicine by the people of Gorontalo, Indonesia. This plant contains water, minerals, carbohydrates, fats, proteins (Ismail et al. 2020), vitamin C (Choong et al. 2016), phenolic and amino acids (Kurniawan et al. 2018). Palm sap (*A. pinnata*) has several pharmacological, including a diuretic, anti-tuberculosis and anti-fatigue. In addition, vitamins can help prevent insulin resistance due to Reactive Oxygen Species (ROS) that damage pancreatic cells (Fitriani et al. 2018). Furthermore, given their antioxidant and antihyperglycemic properties, phenolics aid in the reduction of blood glucose in people with diabetes by preventing excessive oxidation and maintaining insulin content in pancreatic cells, binding free radicals and removing them from the body through the excretory system (Wisudanti 2016). Thus, the content of palm sap (*A. pinnata*) has great potential in treating DM.

Palm sap was applied to experimental animals in the form of male mice (*Mus musculus* Linnaeus, 1758). The mice used were normal mice induced with diabetogenic agents. The use of mice as experimental animals is because mice have a high sensitivity compared to other experimental animals to blood glucose tests, male mice are not influenced by hormonal factors such as female mice. In addition, mice have genes similar to humans (Rudiawan

2016). Therefore, before being given palm sap, the mice were first induced with a diabetogenic agent.

Alloxan, a well-known diabetogenic agent, is widely used to induce type 2 diabetes in animals. In addition, alloxan-induced diabetes mellitus serves as a pathological bio model for testing a substance with supposed antioxidant activities. Alloxan can increase blood glucose in mice within 2 x 24 hours without causing death (Rudiawan 2016).

MATERIALS AND METHODS

Research design

This study employed a post-test control group design, i.e., the effect of treatment, by comparing the treatment group with the control group after being given the action. The experimental design used was a randomized design (CRD) with five treatment groups consisting of two control groups and three groups of palm sap (0.2 mL, 0.4 mL, 0.6 mL).

Sampling and preparation

Palm sap is obtained from the community in Dulamayo, Bone Bolango District, Gorontalo, Indonesia. Male mice (M. musculus) aged 2-3 months were involved as test animals. Alloxan was chosen as the diabetogenic agent since it is the most extensively used chemical for producing diabetes in test animals. Glibenclamide as standard hypoglycemic medication and aquades is utilized as drinking water for mice since it is mineral-free, ensuring that the fall in blood glucose levels is not due to minerals in the water.

Group of test animals

Mice were divided into five treatment groups, those are K- = negative control group only given aquadest and feed, K+ = positive control group given glibenclamide 0.013 mg/20 g mice BW, K+ = positive control group given glibenclamide 0.013 mg/20 g mice BW, K+ = positive control group given glibenclamide 0.013 mg/20 g mice BW, K+ = positive control group given glibenclamide 0.013 mg/20 g mice BW, K P1 = Treatment 1, palm sap was given at a dose of 0.2 mL/20 g BW mice, P2 = Treatment 2, palm sap was given at a dose of 0.4 mL/20 g BW mice, and P3 = Treatment 3, given palm at a dose of 0.6 mL/20 g BW rats.

Research procedure

Test animals preparation (acclimatization)

Mice were acclimatized for seven days to adapt to the new environment and were given water and food ad libitum.

Alloxan treatment to mice

Alloxan was induced subcutaneously at 100 mg/kg BW dosage after acclimation. Fasting blood glucose (FBG) levels were measured on the third day following alloxan induction. Before measurement, mice fasted for 10 hours. This study employed mice with FBG >126 mg/dL.

Test materials provision

On the 4th day, glibenclamide and palm sap were given for three days in a row according to the group, and the dose was determined after alloxan was induced. Next, GCU was used to measure fasting blood glucose levels on day 6, and the mice were fasted for 10 hours before being measured.

Data analysis

The research data were analyzed using SPSS (Statistical Products and Services Solutions) version 25. Normality was assessed using the Shapiro-Wilk method ($p>0.05$), and homogeneity was determined using the Levene method ($p>0.05$) and proceeded with the one-way ANOVA test. If there is a significant difference, it would be continued with Duncan's test to see the difference between the treatment groups.

RESULTS AND DISCUSSION

Results

A total of 25 mice (*M. musculus*) were given three different doses of palm sap (0.2 mL, 0.4 mL, and 0.6 mL) and glibenclamide and distilled water as positive and negative controls, respectively. A total of 3 blood draws were performed to measure blood glucose levels (mg/dL). A post hoc test revealed no significant difference between groups ($p<0.05$) before alloxan induction towards the mice. This is supported by the fact that all groups experienced the same condition. The measurement results of FBG were also within the limits of normal FBG (<126 mg/dL), which shows that there is no hyperglycemic activity in the mice before the administration of any substance. After the third day of alloxan administration, all groups' FBG showed a hyperglycemic condition, and there was no significant difference. FBG increased significantly ($p<0.05$) after alloxan administration to mice compared to before administration. After giving palm sap, hyperglycemia was shown in all of the groups following the decreasing FBG of mice. GDP levels were lower in the palm sap (*A. pinnata*) group (P1, P2, and P3) than in the control group (K- and K+). The data of glucose measurement results can be seen in Table 1 and Figure 1.

Based on the results, the percentage of the palm sap group with a dose of 0.4 mL was more influential in lowering fasting blood glucose in mice after alloxan was induced. An ANOVA statistical test was used to measure if there was a significant effect on each group, and normality and homogeneity checks were performed before the implementation. Based on the normality test results using the Shapiro-Wilk test, the p-value is 0.05, which means the research data is normally distributed. Furthermore, based on the homogeneity test using the Levene test, a p-value of 0.05 was obtained, which means that the fasting blood glucose of mice was homogeneous. Bearing this in mind, the requirements in the ANOVA test are met.

Table 1. Average fasting blood glucose (FBG) levels before and after treatment

Group	Before administration of alloxan ±SD	After administration of alloxan ±SD	After administration of palm sap ±SD	Decrease in FBG after giving palm sap (%)
K- (Without Treatment)	$86.20 \pm 3{,}52^{1,a}$	$139.80 \pm 3{,}59^{2,a}$	$112.80 \pm 3{,}38^{3,a}$	19%
K+ (Glibenclamide)	$85.20 \pm 4{,}64^{1,a}$	$139.60 \pm 5.05^{2,a}$	$101.00 \pm 2.07^{3,a}$	28%
P1 (Palm Sap 0.2 mL)	$81.40 \pm 4{,}92^{1,a}$	$133.40 \pm 3{,}76^{2,a}$	$84. \pm 5.07^{1,b}$	37%
P2 (Palm Sap 0.4 mL)	$82.20 \pm 5{,}28^{1,a}$	$141.80 \pm 5{,}81^{2,a}$	$64.00 \pm 5.32^{3,c}$	55%
P3 (Palm Sap 0.6 mL)	$81.80 \pm 2.17^{1,a}$	$134.40 \pm 4{,}79^{2,a}$	$78.40 \pm 3.26^{1,b}$	42%

Note: Group K- (negative control): alloxan + aquadest, K+ (positive control): alloxan + glibenclamide, P1 (treatment 1): alloxan + palm sap 0.2 mL, P2 (treatment 2): alloxan + palm sap 0.4 mL, P3 (treatment 3): alloxan + palm sap 0.6 mL. The different superscript letters within the same column were significantly different at P < 0.05. The different superscript numbers within the same row were significantly different at P < 0.05

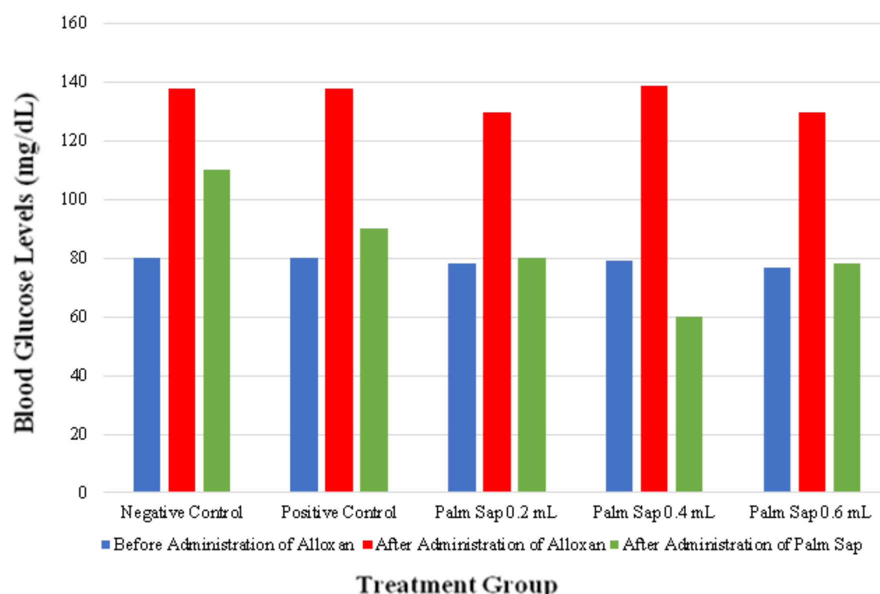

Figure 1. Average fasting blood glucose (FBG) levels in mice before and after treatment

Based on the One Way ANOVA statistical analysis results, there was an effect of giving palm sap on reducing fasting blood glucose in mice with a significant value = 0. 00 = 0.05. To see the difference in each treatment entails Duncan's test. The results of Duncan's analysis showed that the K- group was not significantly different from K+ but significantly different from the P1, P2, and P3 groups. The P1 group was significantly different from the K-, K+, and P2 groups but not significantly different from the P3 group. The P2 group was significantly different from the K-, K+ and P1 groups (Table 1).

The normality test utilizing the Shapiro-Wilk test yielded a p-value of 0.05, indicating that the research data is normally distributed (Appendix 2). Furthermore, the p-value of 0.05 was achieved based on the Levene test's homogeneity test, indicating that the fasting blood glucose of mice was homogeneous (Appendix 2). By this, the ANOVA test's conditions are met. According to the results of a One Way ANOVA statistical analysis, there was an effect of feeding palm sap on reducing fasting blood glucose in mice, with a value of sig = 0. 00 = 0.05. Then, I continued Duncan's test to see how each treatment differed. Duncan's study revealed that the K- group did not differ significantly from the K+ group, but did differ considerably from the P1, P2, and P3 groups. The P1 group differed considerably from the K-, K+, and P2 groups, but not from the P3 group. The P2 group differed significantly from the K-, K+, and P1 groups (Table 1).

Discussion

The measurement of fasting blood glucose (GDP) before alloxan was given, all mice were in normal condition (70-100 mg/dL). However, after alloxan was induced, the levels of FBG in mice experienced hyperglycemia (>126 mg/dL). This result proves that alloxan can produce experimental diabetic conditions in test animals. Alloxan is a widely used chemical to induce diabetes in test animals.

Toxicity produced by alloxan occurs due to the formation of free radicals (superoxide radicals and hydroxyl radicals). Free radical activity causes an increase in cytosolic calcium concentration resulting in pancreatic-

cell necrosis. A decrease follows this damage in insulin secretion, which results in the reaction of glycogenesis and reduced glucose transport in cells. In addition, glycogenolysis becomes uncontrolled, resulting in increased blood glucose in the body (Yusni et al. 2017). Meanwhile, according to Sari et al. (2020), alloxan is a toxic glucose analog that selectively destroys insulin-producing pancreatic cells.

In this study, the K- group was the alloxan induction group but was not given any treatment. The K- group, before being induced with alloxan had fasting blood glucose of 86.20±3.52 mg/dL (normal), after alloxan was induced, fasting blood glucose increased to139.80±3.59 mg/dL (hyperglycemia), then there was a decrease in fasting blood glucose by 19%. This decrease is caused by the body's defense efforts to ward off free radicals. In addition, it is because mice's body has endogenous antioxidants such as catalase, superoxide dismutase (SOD), glutathione peroxidase, and glutathione S-transferase (Pratama and Busman 2020).

The K+ group in this study was the group that was given glibenclamide. The K+ group, before being induced with alloxan had a fasting blood glucose of 85.2±4.64 mg/dL (normal). After alloxan was induced, fasting blood glucose increased to 139.60±5.05 mg/dL (hyperglycemia). Yet, after giving glibenclamide, fasting blood glucose decreased by 28%.

Glibenclamide was administered to the K+ group in this study. Before being induced with alloxan, the K+ group had a fasting blood glucose of 85.24.64 mg/dL (normal), fasting blood glucose climbed to 139.605.05mg/dL (hyperglycemia), and fasting blood glucose was reduced by 28% after being given glibenclamide.

Based on the results of the One Way ANOVA test, the most significant group in reducing fasting blood glucose in mice was the palm sap group with a dose of 0.4 mL (P2) and 0.6 mL (P3), but based on the calculation of the percentage of the palm sap group with a dose of 0.4 mL (P2) which most influential with a decrease of 55%. This decrease was higher than the glibenclamide (K+) group of 28%, 0.2 mL of palm juice (P2) 37% and 0.6 mL of palm juice (P3) 42%. This study is by research conducted by Swastini et al. (2018), which found that the administration of palm sap reduces fasting blood glucose in male rats induced by alloxan.

The decrease in fasting blood glucose in the palm sap group is thought to be influenced by the compounds contained in palm sap which can prevent the oxidation of pancreatic -cells caused by alloxan induction so that the damage can be controlled. The compounds contained in palm sap are minerals, protein (Ismail et al. 2020), vitamin C (Lempang and Mangopang 2012), triterpenoids, flavonoids, saponins, and alkaloids (Putri et al. 2020).

Protein aids in the regulation of high fasting blood glucose in the body. Based on Gannon et al. (2003), protein can increase insulin concentration. According to Prastari et al. (2017), protein plays a role in inhibiting the work of the -glucosidase enzyme in breaking down carbohydrates into glucose, allowing fasting blood glucose to be managed.

Phenolics play a role in lowering fasting blood glucose by damaging the lipid oxidation structure by donating hydrogen atoms (Bahman et al. 2019). In addition, phenolic inhibits enzymes involved in carbohydrate metabolism processes such as -amylase and -glucosidase. Inhibition of this enzyme slows the breakdown of disaccharides into simple glucose so that the body absorbs glucose is reduced (Oboh and Ademosun 2011). The phenolic groups found in palm sap are triterpenoids, flavonoids, saponins, and alkaloids (Putri et al. 2020).

Triterpenoids work as antidiabetics by causing an increase in AMP-activated protein kinase (AMPK) in muscle cells, which enhances glucose consumption in muscle cells and lowers blood glucose. Triterpenoids also help reduce pancreatic-cell damage by delaying the synthesis of TNF- (Tumor Necrosis Factor-alpha) produced by ROS activity (Putra 2013). In insulin-resistant cells, triterpenoids accelerate glucose metabolism and can lower fasting blood glucose in vivo (Hu et al. 2014).

Flavonoids are antioxidants that can inhibit pancreatic cell damage by neutralizing free radicals (Parwata et al. 2018). In addition, flavonoids stop the peroxidation process by inhibiting enzymes that produce superoxide anions and decreasing alkoxyl and peroxyl radicals (Yuslianti 2017). Furthermore, flavonoids act directly on pancreatic -cells, activating the cAMP signal cascade to boost insulin production, triggered by glucose (Hikmah et al. 2016), preventing glucose absorption in the gut (Parwata et al. 2018).

Alkaloids have therapeutic effects and are antidiabetic since these compounds have the potential to interact with various proteins involved in glucose homeostasis (Rasouli et al. 2020). Alkaloids act as antidiabetic, both in extracts and isolated molecules. It inhibited the glucosidase enzyme, deactivated DPP-IV (Dipeptidyl Peptidase-4), improved insulin sensitivity, and reduced oxidative stress (Ajebli et al. 2021)

Vitamin C helps repair damaged cells and insulin resistance and inflexible blood vessels associated with diabetes (Aprila et al. 2015). Vitamin C is a group of micronutrients that plays a role as an antioxidant in human plasma and functions as an electron donor or electron reducing agent, which allows it to serve as an antioxidant. According to Maha (2013), Vitamin C can lower fasting blood glucose, which can provide primary and secondary protection against oxidative damage to lipids and lipoproteins and improve the negative effects of DM.

Aprila et al. (2015) stated that magnesium simplifies glucose transport into cells, cofactors various enzymes involved in the glucose oxidation process, and helps to increase insulin sensitivity and avoids insulin resistance (Larsson and Wolk 2007). In addition, intracellular magnesium deficiency can reduce the action of tyrosine kinase on insulin receptors (Mulatsih 2020).

In conclusion, the administration of palm sap (*A. pinnata*) can lower fasting blood glucose in mice (*M. musculus*) with alloxan-induced diabetes. Palm sap (*A. pinnata*) 0.4 mL was the most significant in lowering the mice's fasting blood glucose.

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Short Communication: Extraction and characterization of pectin from ripe and unripe mango (*Mangifera indica*) peel

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Abstract. *Shaibu CO, Dinshiya J, Shaibu VE. 2022. Short Communication: Extraction and characterization of pectin from ripe and unripe mango (*Mangifera indica*) peel. Asian J Nat Prod Biochem 20: 16-20.* Pectin is a heteropolysaccharide present in the cell walls of different plants at different concentrations with widespread applications. This work aimed at extracting and characterizing pectin from ripe and unripe mango peel to investigate the effect of ripeness on the yield and quality of mango pectin. To obtain the optimum extraction condition, pectin was extracted at varying temperatures, time and pH using 0.1 N HCl as the extraction solvent. The maximum yield of pectin was found to be 22.67% for ripe mango peel and 21.90% for unripe mango peel. The optimum extraction conditions were found to be 90ºC, 60mins and pH 1.5. The pectin extracted using the optimum extraction conditions was then characterized. The moisture content, ash content, methoxyl content, equivalent weight, anhydrouronic acid and degree of esterification of the ripe mango peel pectin were found to be 8.76±0.08%, 10.12±0.47%, 9.17±0.27%, 883.07±13.85 g, 72.45±0.59 and 72.52±0.09% , respectively. In contrast, those of unripe mango peel pectin were found to be 8.13±0.13%, 9.12±0.34%, 8.83±0.19%, 823.38±14.07 g, and 71.56±0.34%, 70.34±0.38%, respectively. This study showed that ripe mango peel might be more suitable for use as a pectin source. However, pectin extracted from ripe and unripe mango peel could be considered an alternative source of pectin in food processing, pharmaceutical industries, and various places of pectin application.

Keywords: Characterization, extraction, mango, pectin, yield

INTRODUCTION

According to research by Singh et al. (2021), over 2 billion tonnes of agro-waste are generated annually worldwide. This volume of waste is capable of causing environmental and health hazards. Therefore, their reuse has been strongly advocated to avert undesirable effects of their accumulation on the environment and maximize benefits from their utilization (Harshwardhan and Upadhyay 2017).

Mango (*Mangifera indica* L.*)* belongs to the family Anacardiaceae with over 1,365 varieties worldwide (Mubarik et al. 2020). Mangoes have been reported to contain high vitamin C, fiber, and pectin (Mubarik et al. 2020). In 2017, Mango production was reported to be about 47 million tonnes per annum (Altendorf 2017). In addition, 40-50% of the fruit constitutes agro-waste (Rai et al. 2020) which can serve as a good source for pectin production.

Pectin belongs to the family of heteropolysaccharides primarily found in the primary cell walls of terrestrial plants (Maxwell et al. 2012; Hamed and Mustafa 2018; Serna and Ayala 2020). It is constituted of 4 polymers rich in D-galacturonic acid (GalA) and containing significant amounts of L-rhamnose (Rha), D-arabinose (Ara), and D-galactose (Gal) (Fissore et al. 2012).

Agro-industrial wastes utilized for pectin production include fruit wastes such as sweet melon (Rahmani et al. 2020), kiwifruit pomace (Yuliarti et al. 2015), papaya peels (Koubala et al. 2014), lemon, grapefruit, orange, avocado, (Bamba et al. 2020), sugar beet (Maxwell et al. 2016).

Pectin is highly valued as a functional food ingredient and has wide pharmaceutical/medicinal applications, including drug delivery, gene delivery, wound healing, and tissue engineering (Maran et al. 2013; Wicker et al. 2014).

Extraction of pectin usually involves hydrolysis of pectin macromolecules under controlled temperature, pH, and time. Temperature, pH, extraction solvent, and extraction time have shown significant effects on pectin yield (Kliemann et al. 2009; Sharma et al. 2013). Acid extraction seems to be the most widely used method, although other methods like the use of enzymes, microwave, subcritical water, and high pressure have been employed in the extraction of pectin (Zoghi et al. 2021)

This study aimed at extracting and characterizing pectin from ripe and unripe mango peel to investigate the effect of ripeness on pectin yield and quality from mango.

MATERIALS AND METHODS

Materials

All the chemicals used were of analytical grade and included Sodium chloride (NaCl), distilled water, ethanol (C_2H_5OH), Hydrochloric Acid (HCl), phenol red, and sodium hydroxide (NaOH).

Equipment

Mortar and pestle, Beakers, Conical flasks, Spatula, Filter paper (Whatman No. 1), Masking tape, Separator funnel, Digital analytical weighing balance, Spectrophotometer (JENWAY: 6305), Refrigerator, Thermostatic Water Cabinet (MODEL: HH - W420), Micropipette, Test tubes and Test Tube Racks, Centrifuge (MODEL: 80-2B), and Sample Bottles.

Sample collection

Both ripe and unripe Mango fruit were collected from Federal University Wukari farmland, Taraba state, Nigeria, located on $7^0$50'34.0″ N $9^0$46'19.1″ E.

Methods

Sample preparation

Ripe and unripe mango fruits (*M. indica*) were peeled, chopped, and blanched in a water bath at 80ºC for 5 minutes. The blanched sample was then oven-dried at 60ºC for 72 hours and ground into fine powder. It was then sieved through a 60mm to 80mm mesh sieve and stored in a separate air-tight container until it was required for further analysis.

Note: The decree of ripeness of the mango used in this study was 4 for unripe and 8 for ripe using a scale of 1 to 10, with 1 as most unripe and 10 as most ripe.

Pectin extraction

Pectin extraction was performed using 0.1 N HCl solvent at different extraction temperatures (70, 80, and 90ºC), extraction times (30, 60 and 90 mins), and pH (1.5, 2.5, and 3.0). 10 g of the powdered ripe mango peel was macerated in 250 mL extraction solvent, and the pH was adjusted with 0.1 N HCl and NaOH. Afterward, the mixture was heated in a water bath for the specified time. The mixture was filtered through a cheesecloth and pressed to recover the extract, pectin was then precipitated from the mixture using 98% ethanol in the ratio of 1:2 (1 part extract and 2 parts ethanol) kept at room temperature overnight. The precipitated pectin was then filtered through Whatman No.1 (filter paper) and washed with 70% ethanol (v/v) and 80% ethanol (v/v) to remove the soluble impurities. The extracted pectin was then dried at 60ºC for 24 hours in an oven. The same procedure was repeated for unripe mango peel.

Determination of % yield

Pectin yield was calculated using the formula:

$$\frac{\text{Weight of pectin}}{\text{Weight of sample used}} \times \frac{100}{1}$$

Pectin characterization

Ash content: Ash content of pectin was determined by Ranganna's method (Ranganna 1986). A 1.2 g pectin (sample) was weighed, the sample was ignited slowly in a crucible and then heated for 3-4 hours at 600ºC, then the crucible was taken into the desiccators and allowed to cool.

Ash content was determined using the equation:

$$\text{Ash\%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

Moisture content: The moisture content of pectin was determined by Ranganna's method (Ranganna 1986). A 1 g pectin sample was weighed and placed into a metal dish. The sample was dried in an oven for 5 hours at 100°C and then cooled in a desiccator and weighed.

The moisture content was determined using the equation:

$$\text{Moisture content (\%)} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100$$

Equivalent weight: Equivalent weight was determined by Ranganna's method (Ranganna 1986). A 0.5 g pectin was measured into a 250 mL conical flask, and 5 mL ethanol was added, 1 g of sodium chloride and 100 mL of distilled water were added to the solution, after which 6 drops of phenol red were added. The mixture was titrated against 0.1 N NaOH. A purple color indicated the titration endpoint. The neutralized solution was stored for the determination of methoxyl content and anhydrouronic acid content.

$$\text{Equivalent Weight} = \frac{\text{Weight of sample} \times 1000}{\text{ml of alkali} \times \text{Normality of alkali}}$$

Methoxyl content (MeO): The methoxyl content is an important factor in controlling the setting time of pectins. MeO was determined using Ranganna's method (Ranganna 1986). First, the 25 mL sodium hydroxide (0.25 N) was added to the neutral solution obtained from the determination of equivalent weight. Next, the solution was stirred thoroughly and kept at room temperature for 30 mins. After 30 min, 25 mL of 0.25 N hydrochloric acid was added and titrated against 0.1 N NaOH.

Methoxyl content was calculated by using the following formula:

$$\text{Methoxyl content\%} = \frac{\text{ml of alkali} \times \text{Normality of alkali} \times 3.1}{\text{Weight of sample}}$$

Total anhydrouronic acid content (AUA): Total AUA of pectin was obtained mathematically using the following formula:

$$\text{AUA} = \frac{176 \times 0.1z \times 100}{W \times 1000} + \frac{176 \times 0.1y \times 100}{W \times 1000}$$

Where: Molecular unit of AUA (1 unit) = 176 g, z = mL (titre) of NaOH from equivalent weight determination, y = mL (titre) of NaOH from methoxyl content determination and w = weight of sample.

Degree of esterification (DE)

The DE of pectin was measured based on methoxyl content (MeO) and AUA and calculated using the formula:

$$\text{Degree of esterification (DE) \%} = \frac{176 \times \%\,\text{MeO}}{31 \times \%\text{AUA}} \times 100$$

Statistical analysis

All data are represented as means ± SD. Statistical analyses were performed using the Independent samples t-test on Statistical Package for Social Sciences (SPSS) version 20.

RESULTS AND DISCUSSION

Percentage (%) yield of pectin at pH 1.5, 60 minutes, and varying temperature

The percentage yield of pectin at 60 mins, pH 2.0 and different temperature is shown in Figure 1. The percentage yield of pectin increased with increase in temperature for both mango peels, pectin from the ripe mango was shown to be higher than that of the unripe mango peel.

Percentage (%) yield of pectin at 90°C, 60 minutes, and varying pH

The percentage yield of pectin at 60 mins, temperature 90ºC, and different pHs of 1.5, 2.0, and 3.0 are shown in Figure 2. Pectin yield for ripe and unripe mango peel decreased with an increase in pH. The yield of the ripe mango peel was found to be higher than that of the unripe mango peel.

Percentage (%) yield of pectin at 90ºC, pH 1.5, and varying time

Figure 3 shows the percentage yield of pectin at 90ºC, pH 1.5 and different times. There was an increase in pectin yield for both ripe and unripe mango peel from 30 mins to 60 mins extraction time. However, there was a decrease from 60 mins to 90 mins. The pectin yield of the ripe mango peel was higher when compared to that of the unripe mango peel.

Discussion

Pectin yield

This study considered pectin extraction from ripe and unripe mango peel to investigate the effect of ripeness on the yield and quality of pectin produced from mango. To obtain the optimum extraction condition, pectin was extracted at various temperatures (70ºC, 80ºC, and 90ºC), pH (1.5, 2.0, and 3.0), and time (30 mins, 60 mins, and 90 mins). The optimum extraction conditions shown in this study were pH 1.5, 60 mins extraction time, and 90ºC. Pectin extracted using the optimum extraction condition was used in characterization. Studies on the effect of pH, temperature, and time showed that the total yield of pectin for both ripe and unripe mango peel increased with a decrease in pH from pH 3.0 to pH 1.5. This agrees with the work of Hamidon and Zaidel (2017), who reported that the yield of pectin increased with a decrease in the pH of the extractant while working on potato peel residue. Pectin yield also increased with an increase in extraction time, however, further increasing the extraction time to 90mins showed a decrease in pectin yield. Thus, the optimum time of the extraction for the maximum yield of mango peel pectin was found to be 60 mins. Similar results were reported on pectin extracted from mandarin orange peels by Koubala et al. (2008). The effect of extraction temperature on the yield is shown in Figures 2 and 3. The yield of pectin increased significantly with the increase in extraction temperature. Similar observations were reported for dried mango peel (Sangheetha et al. 2018).

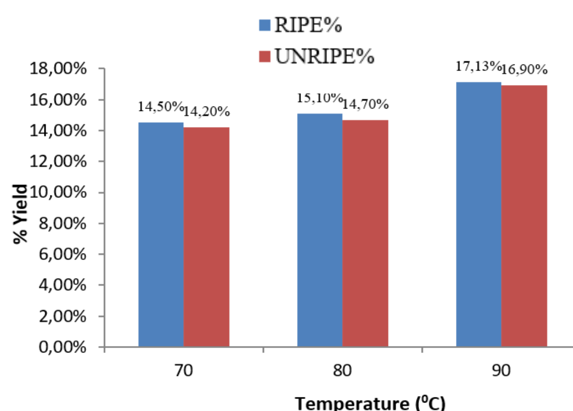

Figure 1. % Yield of pectin at pH 1.5, 60 minutes, and varying temperature

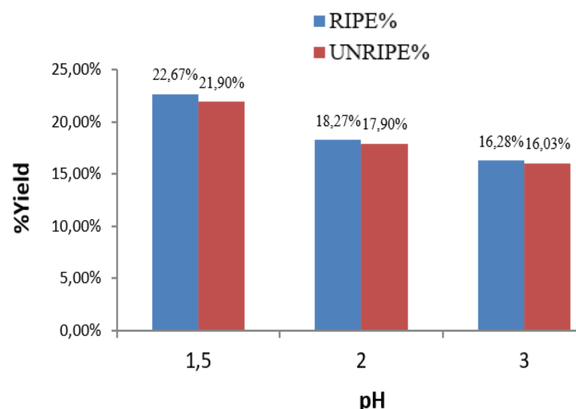

Figure 2. % Yield of pectin at 90°C, 60 minutes, and varying pH

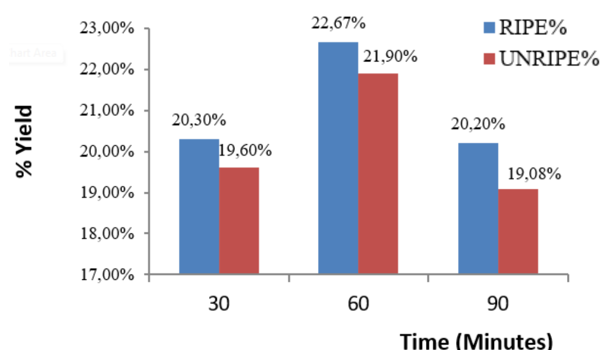

Figure 3. % Yield of pectin at 90ºC, pH 1.5, and varying time

The maximum yield was 22.67% and 21.94% for ripe and unripe mango peel, respectively. Higher pectin yield for ripe mango peel may result from a higher concentration of pectin due to the maturation of the fruit. On the other hand, pectin concentration may decrease with an increase in ripeness due to pectin hydrolysis by pectinase (Prasanna et al. 2007). This is as opposed to the findings of Castillo-Israel et al. (2015), who had higher values for pectin yield from an unripe banana than from the ripe banana peel. However, the yield of pectin from both ripe and unripe mango peel compares well to the 2.93-25.80% pectin yield recorded by Bamba et al. (2020) from orange, lemon, grapefruit, and avocado and is higher than those reported by Yapo and Koffi (2006), for passion fruit pectin (7.5%), and ambarella peel pectin (10-13%).

Characterization of mango peel pectin

Ripe mango pectin had significantly higher values for yield moisture content, ash content, equivalent weight, and degree of esterification (Table 1). There was no significant difference in methoxyl content and anhydrouronic acid. The moisture content of ripe and unripe mango peel pectin compares well with 10.59 and 11.68 reported for mango peel and watermelon pectin, respectively, by Rury et al. (2017). However, the moisture content of the unripe mango pectin was significantly lower than that of the ripe mango peel.

Ash content measures the total amount of minerals present within a food. The ash content of the pectin isolated in this work was found to be as high as 9-11%. Nazaruddin et al. (2011) reported that the maximum value needed for the quality gel is 10%. This shows that pectin extracted from both mango peels could be a good source for producing quality gel.

There was no significant difference in the methoxyl contents of pectin extracted from ripe and unripe mango peel. Methoxyl content of commercial pectins generally varies from 8-11% and can form high sugar gels (>65% sugar) (Castillo-Israel et al. 2015). Therefore, this study's values obtained from ripe and unripe mango peel compare favorably with commercially available pectin.

The extracted pectin's equivalent weight was 883.07±13.85 g and 823.38±14.07 g for ripe and unripe mango peel, respectively. However, this indicates that the ripe mango pectin has a significantly higher gel-forming ability than the unripe. Values obtained in this study were lower than those of apple pomace pectin (833.33-1666.30), Kumar and Chauhan (2010), but higher than those of cocoa husk pectin (510.68-645.19), as reported by Ramli and Asmawati (2011).

Anhydrouronic acid (AUA) is essential to determine the purity and degree of esterification and evaluate physical properties (Ranganna 1986). It indicates the purity of extracted pectin. Pectin with an AUA of 65% or more is considered pure (Nazaruddin et al. 2011). Values for AUA (%) obtained in this study are greater than 65%, indicating that they are pure.

The degree of esterification (DE) obtained in this study was within the 60-90% range, generally found in plant tissues (Shaha et al. 2013). Pectins could be classified as rapid-set (DE >72%) and slow-set (DE: 58-65%). This describes the rate of gel formation. Pectin from ripe and unripe mango peels had DE values less than 75% but greater than the range for slow-set pectins, this shows that they may be classified as rapid-set pectin. Values obtained were lower than those reported by Wang et al. (2014), who reported DE values of 76.30 and 83.41% in pectin from the fruit peel of *Citrus maxima* and apple pomace, respectively.

Table 1. Pectin characterization

Parameter	Ripe (%)	Unripe (%)	^{A} Mean differences	P values of mean differences
Yield	22.67	21.90	0.77	0.002^{c}
Moisture content	8.76±0.08	8.13±0.13	0.63	0.021^{c}
Methoxy content	9.17±0.27	8.83±0.19	0.34	0.668^{b}
Ash content	10.12±0.47	9.12±0.34	1.00	0.026^{c}
Equivalence weight	883.07±13.85g	823.38±14.07	59.69	0.003^{c}
Anhydrouronic acid	72.45±0.59	71.56±0.34	0.89	0.148^{b}
Degreee of esterification	72.52±0.09	70.34±0.38	2.18	0.007^{c}

Note: Values represent Mean ± standard deviation. ^{a}= indicates the mean values of parameters for ripe seeds minus the mean values of unripe ones. ^{b}= Mean difference is not significant ^{c}=Mean difference is significant at P<0.05

The equivalent weights of the extracted pectin were found to be high, indicating that they have a low partial degradation property. The degree of esterification, equivalent weight, and ash content of ripe mango pectin were significantly higher than that of the unripe mango peel pectin. There was no significant difference in methoxyl and anhydrouronic acid content, thus indicating that the quality of pectin obtainable from ripe mango peel is comparatively better than pectin obtained from unripe mango peel in terms of gel formation and purity. However, both ripe and unripe mango peel pectin is comparable to apple pomace and citrus, thus, indicating the possible use of mango peel as an alternative source in commercial pectin production and for industrial, pharmaceutical, medical, and agricultural applications.

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Extraction, purification, and quantification of hesperidin from the immature *Citrus grandis/maxima* fruit Nepal cultivar

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Abstract. *Manikyam HK, Tripathi P, Patil SB, Lamichhane J, Chaitanya MVNL, Patil AR. 2022. Extraction, purification, and quantification of hesperidin from the immature* Citrus grandis/maxima *fruit Nepal cultivar. Asian J Nat Prod Biochem 20: 21-26.* Hesperidin, a flavanone group of flavonoids and a 7-O-rutinoside of hesperitin is abundantly present in citrus fruits. Pomelo or *Citrus grandis* (L.) Osbeck/ *C. maxima* (Burm.) Merr. is grown and cultivated in Nepal as seasonal edible fruit. Pomelo has flavanone and other chemical constituents. Flavanones like naringin, hesperidin, neohesperidin, and naringenin are present in the fruits of *C. grandis*. Many extraction techniques have been reported in the extraction and purification of hesperidin citrus fruits like sweet lemons, grapefruits, mandarins, etc. However, little information has been provided on hesperidin content in pomelo fruits. In order to estimate the quantity of hesperidin in the pomelo fruits of Nepal, we have first time made study. The immature pomelo fruits of size 2-7 cm were collected from the cultivation field and dried completely. The pulverized fruit powder with moisture content less than 15% was subjected to 5% methanolic acetic extraction and Dimethylformamide (DMF) solvent extraction in Soxhlet apparatus by refluxing at 90°C in 1:15 ration in three subsequent extractions of each 1 hour. The concentrates were crystalized at freezing temperatures in methanol and further purified by repeating the crystallization process. The white amorphous powder was subjected to RP-HPLC analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15 cm x 5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.12 g/100 g with acetic acid methanol with a purity of 87% and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction and 90% in DMF extraction when compared with standard 93%. Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus we found DMF and 5% methanolic acetic solvents as preferable solvents to extract hesperidin.

Keywords: Acetic acid, *Citrus grandis/maxima*, dimethylformamide, hesperidin, methanol, Nepal cultivar, RP-HPLC UV method

INTRODUCTION

Citrus grandis (L.) Osbeck/ *C. maxima* (Burm.) Merr. commonly called pomelo (red pomelo), is an important member of the Rutaceae family, well known for its high morphological variability, nutritional and commercial values (Alam et al. 2014; Susandarini et al. 2020). It was found in Barbados and commercially grown in Morocco, Israel, Spain, South Africa, Brazil, USA, and some Asian countries (Jokic et al. 2019). In Nepal, it is called Bhogate and is eaten as seasonal fruit. In Nepal, they make Bhogate sadheko, a common salad eaten by local people. In India commonly called Chakotra. China is the major producer of pomelo, which is more than 60% of the world's cultivation (Man et al. 2015). Many bioactive compounds like flavanones, flavones, flavonols, and anthocyanin are abundantly present in the pomelo fruit. Chemical ingredients like limonoid glycones and aglycones, coumarins, naringin, hesperidin flavanones, etc. are the most abundant compounds in fruits of pomelo. Naringin is the most abundant flavanone present, around 295 to 377 mg/L in grapefruits and pomelo. many research studies have proved that the extracts of pomelo have a therapeutic effect on cancer, inflammation, diabetes, neurodegenerative and cardiovascular diseases with powerful antioxidant activity (Damon et al. 1987; Ma et al. 2008). It was studied that active components in the pomelo extract by using high-performance liquid chromatography (HPLC) had naringin (11.90 ± 0.21 mg/g dried extract), hesperidin (12.04 ± 0.12 mg/g dried extract), neohesperidin (25.4 ± 0.12 mg/g dried extract), and naringenin (9.20 ± 0.19 mg/g dried extract) (El-Shafae and El-Domiaty 2001; Lucker et al. 2002; Iglesias-Carres et al. 2019).

Hesperidin bio-flavanone, also known as hesperitin 7-rutinoside or 7-O-glycoside hesperitin (3,5,7 trihydroxyfavanone 7-rhamnoglucoside, C28H34O15). Figure 1, abundantly present in citrus fruits with a molecular weight of 610.57 with melting point 250-255°C and boiling point of 576.17°C, respectively. It is not soluble in water and highly soluble in alkaline solutions but

moderately soluble in propylene glycol and Poly ethylene glycol 400 (Kanaze et al. 2004). It is highly soluble in DMSO and DMF. Poorly soluble in solvents like ethanol, isopropanol, and methanol (when purified) (Pandey et al. 2015).

Hesperidin is generally considered safe for topical and systemic administrations with 2% in topical applications, it has shown no adverse cutaneous reactions in mice for 9 days of administration (Xiong et al. 2019). In addition, the oral formulation Daflon-500 mg containing 10% hesperidin with a daily dosage of 100 mg showed no adverse effects in rats (Razavi and Hosseinzadeh 2019).

Multiple therapeutic effects of hesperidin include anti-inflammatory, lipid-lowering, analgesic, antioxidant, and anticancer activities. Other than mentioned health benefits, hesperidin is well known to treat venous insufficiency, varicose veins, venous ulcers, and hemorrhoids (Paudyal and Haq 2008; Man et al. 2019). By stimulating the release of the appetite-regulating hormone cholecystokinin (CCK) in enteroendocrine STC-1 cells, both hesperitin and hesperidin help treat obesity by controlling appetite (Magwaza et al. 2015). In one of the studies on the neuroblastoma cell line, hesperidin has exerted a neuroprotective effect against rotenone by its antioxidant effect and maintaining mitochondrial functioning (Wu et al. 2007).

In recent studies, attempted extraction of hesperidin from peels of *Citrus reticulata* Blanco mandarin using deep eutectic solvents (DESs), a sustainable green extraction technique using choline chloride:acetamide (1:2) and water DESs had shown most efficient extraction of hesperidin 112.14 mg/g (Hendrickson and Kesterson 1954). Extraction of hesperidin by ultrasound assistance has shown that solvent has a profound effect on the yields, along with an increase in temperature and extraction time (Kim and Lim 2020). The highest flavonoid yields were achieved by 58.4% ethanol concentration at 80°C, 40 mL/g solvents to feed concentration for 30 minutes, and hesperidin yields of 66.6%. The extraction conditions for the highest flavonoid yields based on a response surface methodology were 80.3°C, 58.4% (ethanol concentration), 40 mL/g (solvent/feed), and 30 min (Tamilselvam et al. 2013). Ethanol extraction was found to be more effective when compared to methanol (yield 57.3%) and acetone (yield 37.7%) (Tamilselvam et al. 2013). Some studies showed that the yield of hesperidin was higher in 70% methanol than in 80% ethanol at 35°C from mandarin peels (Caengprasath et al. 2013), and *C. sinensis* pulp was higher in 90% methanol than 90% ethanol at 55°C (Kim and Lim 2020).

In the present study for the first time, for the first time, we are extracting hesperidin from immature fruits of the *C. grandis/maxima* (pomelo) cultivar of Nepal and quantifying it.

MATERIALS AND METHODS

Plant material preparation: *C. grandis/maxima* fruits were obtained from the local cultivation field Chisapani area, Kathmandu, Nepal, and immature fruits of size 2-7 cm see Figure 2, were dried till moisture content reached 15%. The 200 g of dried immature fruits of 2-7 cm were pulverized to 180 microns mesh size.

Extraction of hesperidin using acetic acid methanol solvent

Weight separately 100 g of dried immature fruit powder of 2-7 cm size and place it in Soxhlet apparatus with 5% acetic in methanol (1:15 feed/solvent) and continued extraction for 1 hour at 100°C with a circulation of solvent. Total 3 extractions were carried out at an interval of each 1 hour with 1:15 feed to solvent ratio consequently. The total extract is collected, filtered, and concentrated to 80% before chilling. The 80% reduced liquid concentrate was chilled at 5°C for a few hours and filtered. The precipitate of hesperidin is filtered and washed with pure methanol till white-colored amorphous powder appears and is further quantified.

Extraction of hesperidin with dimethylformamide

Weight separately 100 g of dried immature fruit powder of 2-7 cm size and place it in maceration apparatus in DMF (1:15 feed/solvent) and continued extraction stirring for 1 hour at 100°C with a solvent circulation. Total 3 extractions were carried out at an interval of each 1 hour with 1:15 feed to solvent ratio consequently. The extract is collected, filtered and concentrated to 80% before chilling. The 80% reduced liquid concentrate was chilled at 5°C for a few hours and filtered. The precipitate of hesperidin is filtered and washed with pure methanol till white-colored amorphous powder appears and is further quantified.

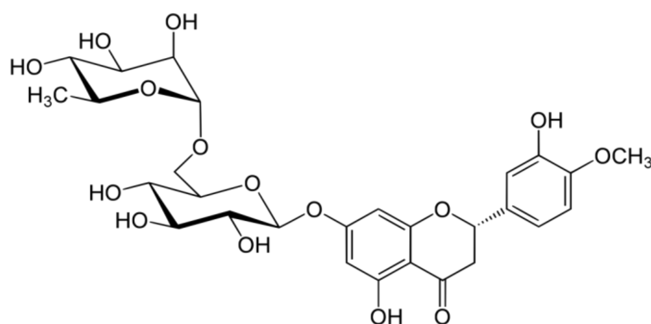

Figure 1. Structure of hesperidin

Figure 2. Immature fruit (2-7 cm) of *Citrus grandis/maxima*

Methods

Loss on drying

Accurately weigh 1.0~2.0 g of sample, and spread it on a dry flat weighing bottle previously weighed. Dry the bottle at 135 ± 2°C for 4 hours. Calculate loss on drying by the following formula, which should be less than 5.0%.

Loss on drying%= $(M1 - M2/M1 - M)$ X100%

Where:

M: Tare weight of the weighing bottle, g;

M1: Weight of the weighing bottle and sample before drying, g;

M2: Weight of the weighing bottle and sample after drying, g

Assay (RP-HPLC)

Chromatographic system

Chromatography system: HPLC with UV detector; Column: 4.6 mm x 15 cm x 5 µm packing C18; Wavelength: UV 284 nm; Flow rate: 1.2 mL/min; Column temperature: 40°C; Injection volume: 10 µL; Run time: 30 minutes

Mobile phase: Methanol: water: Glacial acetic Acid: acetonitrile= 28:66:6:2(V/V), diluent for sample preparation: dimethyl sulphoxide

Preparation of solution

Test solution: Accurately weigh 25 mg of sample to a 50 mL volumetric flask, dissolve and dilute to exactly 50 mL with dimethyl sulphoxide, and mix well.

Reference solution: Accurately weigh 25 mg of a hesperidin reference standard to a 50 mL volumetric flask, dissolve and dilute to exactly 50 mL with dimethyl sulphoxide, and mix well.

Test procedure

Inject reference solution, test solution, and record the chromatograms. Calculate the assay of hesperidin according to a standard external method based on an anhydrous basis. The result of the assay should be not less than 90%.

Assay (%w/w) = (A_T/A_R X W_S/W_R) X P X 100%/1-LOD

A_T : Main peak area in the chromatograms obtained with test solution;

A_R : Main peak area in the chromatograms obtained with reference solution;

W_S : Weight of sample, mg;

W_R : Weight of reference standard, mg;

P : Assay of reference standard, %;

LOD : Loss on drying of sample, %.

Method validation

P Parameters like linearity, precision, accuracy, and limits of detection (LOD) and quantification (LOQ) were developed to determine the hesperidin content by HPLC method. Calibration graphs for linearity were determined using standard solution preparation of hesperidin at different concentrations of 20, 60, and 100 µg/mL. The dilutions were injected in series, and the peak area was calculated for each concentration plotted against the peak area. By adding standard at three concentrations of 20, 60, and 100 µg/mL, percentage recovery was calculated for each concentration to determine accuracy. Hesperidin was injected three times on the same day and on 3 different days to determine intra-day and inter-day variations to determine system precision. Related standard deviation (RSD) was considered a precision measure in both cases. By determining the LOD and LOQ, sensitivity was evaluated. The amount of analyte which gives a peak with a signal-to-noise ratio of 3 was defined as LOD, and the lowest amount of analyte with a signal-to-noise ratio of 10 was considered LOQ. The International Conference on Harmonization (ICH) was considered to validate the method.

Quantification of hesperidin in concentrated methanolic acetic acid extract

The total methanolic acetic acid extracts thus collected after 3 subsequent extractions were combined and concentrated. The concentrated extract was then subjected to RP-HPLC chromatography as mentioned in the assay method for standard hesperidin, and the area of peak in correlation to the retention time of standard was analyzed, and the amount of hesperidin was calculated from the calibration plot obtained by a regression equation.

RESULTS AND DISCUSSION

Hesperidin a bioflavonoid present in all citrus fruits. It is also known as Hesperitin 7-rutinoside or 7-O-glycoside hesperitin (3, 5, 7 trihydroxyfavanone 7-rhamnoglucoside. It is a highly economically viable phytochemical in demand for the Pharmaceutical, Nutraceutical, and cosmeceutical industries. It is a precursor molecule to synthesize Diosmin, a molecule used to treat neurological problems. Extraction of hesperidin using economically viable methods is needed in the present scenario looking at the abundant availability of raw material resources and market demand. Most of the immature citrus fruits varying sizes of 2 mm- 3 mm contain 10-12% of hesperidin. Pomelo (*C. grandis/maxima*) is abundantly grown in Nepal as edible fruit. Pomelo fruit contains vitamin C, beta carotene, anthocyanin, hesperidin, and Naringin ingredients. Even though hesperidin content is found to be less in fruits of pomelo, we attempted to quantify hesperidin in immature dried fruits for the first time in the Nepal cultivar of pomelo. Many works have suggested using ethanol and methanol as solvents to extract hesperidin. In our present work, we have used two solvent methods like 5% acetic acid in methanol and dimethylformamide, to extract hesperidin. The pH and temperature directly affect the recovery of the extraction of hesperidin. In experiments conducted by Hendrickson and Kesterson Florida horticulture society in 1955, they found hesperidin poor solubility in high pH alcoholic solvents and high solubility in low pH alcoholic solvents, and also, the

optimal temperature for extraction should be from 90°C to 100°C (Alam et al. 2014). Research shows hesperidin solubility in DMF and DMSO (30 mg/mL) and soluble in ethanol and methanol (~1 mg/mL). Two separate experiments were carried out to extract hesperidin using 5% acetic acid in methanol and DMF in 1:15 feed to the solvent ratio for 3 hours consecutively at 100°C while stirring, later the liquid extract was filtered and concentrated up to 80%. The concentrate chilled at 5 °C for a few hours. Thus, the precipitate was filtered and further purified by washing it with pure methanol. The obtained hesperidin was quantified using RP-HPLC UV analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15cm x 5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.12 g/100g with acetic acid methanol with a purity of 87% and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction and 90% in DMF extraction when compared with standard 93% see Figure 3 and 4. Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus we suggest using acetic acid methanol, methanol, or DMF as good solvents to extract high yields of hesperidin with good purity.

Method validation

Linearity, LOD, and LOQ

It was confirmed that the linearity of the method for concentrations ranging from 20 to 100 µg/mL of hesperidin. Good linear regressions observed with calibration curves y= 0.3456x + 0.1573 R^2= 0.9973 (Figure 5 and Table 1). The minimum amount of analyte plant extract LOD used for detection and LOQ was 6.66 µg/mL and 20.19 µg/mL, with a retention time of 7.45 minutes.

Precision and accuracy

The precision and accuracy tests were performed by injecting the hesperidin sample three times within the same and three consecutive days. Inter-day and Intra-day precision by RSD of the hesperidin peak areas ranged from 0.5-2.1, which is 2.5% under the limit as per ICH guidelines. By calculating recovery %, accuracy was determined, and for hesperidin, it ranged between 89.99-102%, and the method was proven accurate (Table 2).

Table 1. Calibration data for the proposed HPLC method

Concentration (µg/mL)	Area (10^5 mAU)
20	7.1
40	13.89
60	21.08
80	28.25
100	33.47
120	42.3

Table 2. Recovery and standard deviation data by HPLC

Compound	Amount added (µg/mL)	Amount recovered (µg/mL)[a]	Recovery (%)[a]	RSD (%)	
				Intra day	Inter day
Hesperidin	40	37.09 ± 0.26	92.75± 0.26	1.73	1.879
	80	81 ± 0.26	101.25 ± 0.26	2.12	0.156
	100	98.9 ± 1.69	98.9 ±1.69	0.65	0.48

Note: [a] Mean ± SD (n=3) mean the sample analyzed three times; [b] Samples were analyzed three times a day; [c] Sample were analyzed once a day over three consecutive days

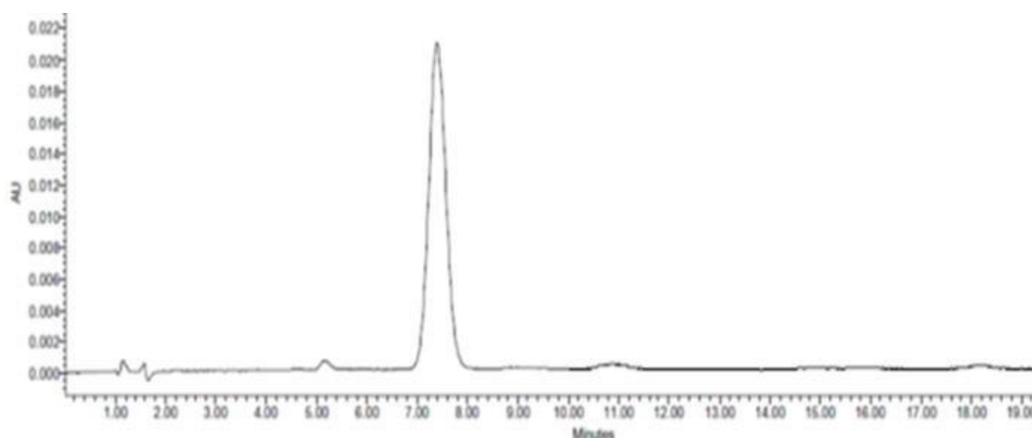

Figure 3. Chromatogram of hesperidin standard 93%

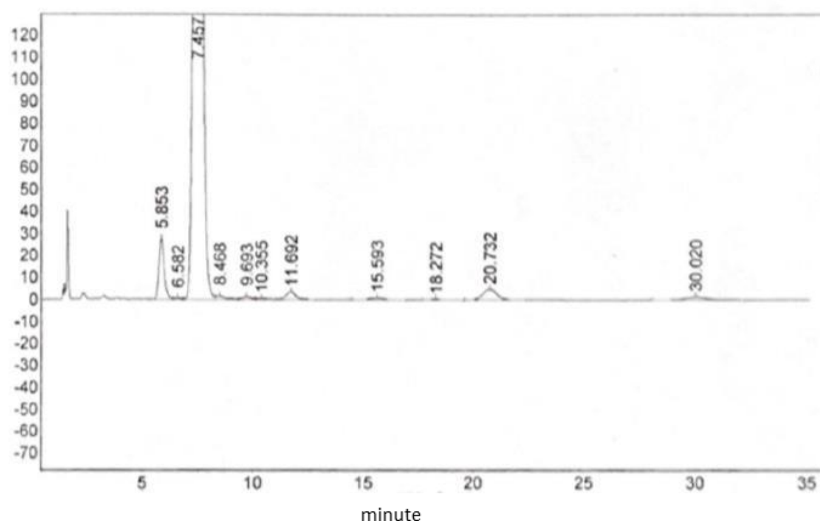

Figure 4. Chromatogram of hesperidin (90% pure) extracted from dried whole immature fruit of *C. grandis/maxima*

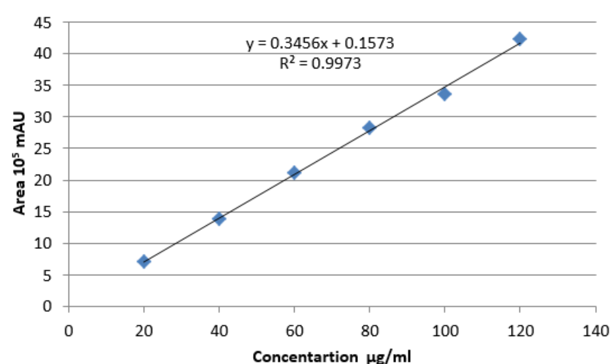

Figure 5. Linearity of hesperidin standard

Discussion

Hesperidin flavanone glycoside is usually found in citrus fruits, and its aglycone is called hesperitin. Plants produce it as a defense mechanism. Different extraction techniques were used earlier to isolate hesperidin with significant yields from different varieties of citrus fruits. It has been found that classical solvent extraction of hesperidin is preferred. The highest flavonoid yield was achieved by 58.4% ethanol concentration at 80°C, 40 mL/g solvents to feed concentration for 30 minutes, and hesperidin yields of 66.6%. The extraction conditions for the highest flavonoid yields based on a response surface methodology were 80.3°C, 58.4% (ethanol concentration), 40 mL/g (solvent/feed), and 30 min (Tamilselvam et al. 2013). Ethanol extraction was found to be more effective when compared to methanol (yield 57.3%) and acetone (yield 37.7%) (Tamilselvam et al. 2013). Some studies showed that the yield of hesperidin was higher in 70% methanol than in 80% ethanol at 35°C from mandarin peels (Caengprasath et al. 2013) and *C. sinensis* pulp was higher in 90% methanol than 90% ethanol at 55°C (Kim and Lim 2020). In our present work, we first time tried to extract hesperidin and quantify it using DMF and 5% methanolic acetic acid as solvents from the whole dried immature fruit of *C. grandis/maxima* of the Nepal cultivar variety. Two separate experiments were carried out to extract hesperidin using 5% acetic acid in methanol and DMF in 1:15 feed to the solvent ratio for 3 hours consecutively at 100°C while stirring, later the liquid extract was filtered and concentrated up to 80%. The concentrate chilled at 5°C for a few hours. Thus, the precipitate was filtered and further purified by washing it with pure methanol. The obtained hesperidin was quantified using RP-HPLC UV analysis method, chromatographic conditions 285 nm wavelength, C18 column 4.6 mm x 15 cm x5 µm, flow rate 1.2 mL/minute with injection volume 10 µL run time of 30 minutes, column temperature 40°C. Hesperidin yields were 0.09 g/100 g with acetic acid methanol with a purity of 87% and 0.17 g/100 g in DMF extraction with a purity of 90% with recoveries of 87% in acetic acid methanol extraction and 90% in DMF extraction when compared with standard 93% see Figures 3 and 4. It was confirmed that the linearity of the method for concentrations ranging from 20 to 100 µg/mL of hesperidin. Good linear regressions observed with calibration curves y= 0.3456x + 0.1573, R^2= 0.9973 (Figure 5 and Table 1). The minimum amount of analyte plant extract LOD used for detection and LOQ was 6.66 µg/mL and 20.19 µg/mL, with a retention time of 7.45 minutes. The precision and accuracy tests were performed by injecting the hesperidin sample three times within the same and three consecutive days. Inter-day and Intra-day precision by RSD of the hesperidin peak areas ranged from 0.5-2.1, which is 2.5% under the limit as per ICH guidelines. By calculating recover %, accuracy was determined, and for hesperidin, it ranged between 89.99-102%, and the method was proven accurate (Table 2).

Further extraction does not yield any hesperidin content indicating a total of 0.15-0.17 g/100 g of hesperidin content in the whole immature fruit of *C. grandis/maxima*. Thus, we suggest using acetic acid methanol, methanol, or DMF as good solvents to extract high yields of hesperidin with good purity.

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