Adetayo *et. al.* BUMJ 2020 3(2):10-18
https://doi.org/10.38029/bumj.v3i2.38

RESEARCH ARTICLE OPEN ACCESS

# In-vitro antisickling and sickling-reversal activities of Carica papaya fruit at different stages of ripening

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**Abstract**
**Objective**: Sickle cell disease (SCD) is a haemoglobinopathy that causes several clinical complications. Unripe *Carica papaya* has been shown to possess antisickling activity that could reduce these complications. This study aimed to examine the presence of antisickling and sickling-reversal activities of aqueous extracts and ethyl acetate fractions of *C. papaya* fruit at different stages of ripening.
**Methods**: Unripe, partly ripe and fully ripe fruits were quantitatively screened for some phytochemicals using standard methods. Blood samples from sickle cell patients were used to investigate antisickling and sickling reversal activities of aqueous extracts and ethyl acetate fractions of the fruits. Data were analysed using one-way ANOVA. p-value was set at 0.05.
**Results**: Phytochemicals such as alkaloids, saponins, tannins, flavonoids and polyphenols were found in varying concentrations in *C. papaya* fruit at the different stages of ripening. All extracts and fractions showed antisickling and sickling-reversal activities with the ethyl acetate fraction of partly ripe *C. papaya* being most effective.
**Conclusion**: The study showed that *C. papaya* fruit at different stages of ripening contains antisickling and sickling-reversal activities which may help reduce the associated complications of SCD when consumed by affected individuals.

**Keywords:** Anti-sickling, Sickling-Reversal, Phytochemical, Carica papaya

## Background

Sickle-cell disease (SCD) is a genetic disorder that occurs due to the presence of abnormal hemoglobin (HbS) in red cells which is caused by the replacement of glutamic acid with valine. This results in the transformation of the bi-convex shape of red blood cells into sickle shape under certain unfavorable conditions including hypoxia, stress, and dehydration (1). It is a multisystemic disease characterized by the premature breakdown of red blood cells leading to constant anemia and vaso-occlusion which results in severe body pains and other manifestations. Sickled erythrocytes tend to block capillaries, causing stasis, and thereby starve organs of both nutrients and oxygen and eventually cause hypofunction or complete tissue destruction (2).

Management of SCD involves the use of medications like hydroxycarbamide, also known as hydroxyurea, reduces the frequency of painful crises, and may also reduce the need for blood transfusions by stimulating the production of fetal hemoglobin (3). Analgesics, antimalarial, antibiotics and antioxidants are also commonly used as supportive drugs (4). Research into the efficacy of plants in the management of SCD has evolved. A commonly used herbal drug with antisickling properties, Niprisan, is essentially plant-based (5). More recently, studies (6, 7) have shown that phytomedicines are very effective as antisickling and sickling-reversal agents.
Research on the antisickling activities of two ethnomedicinal plant recipes used in the treatment of SCD in Ibadan, Nigeria, showed that the methanol extracts of the powdered plant parts

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displayed antisickling activities in vitro. Confirmation of the antisickling activity in the two recipes justified their use in the treatment of the disease. Phytochemical screening of the extracts showed that they contained similar bioactive compounds and each recipe was composed of several plant species, including *C. papaya* (8).

*C. papaya* Linn, commonly known as Pawpaw, is a tropical fruit belonging to the plant family *Caricaceae* and the genus *Carica* native to Mexico and Central America. Different parts of the *C. papaya* plant such as the seeds, leaves, pulp, stem, bark, and root have been used for medicinal purposes and as nutraceutical fruits in the management of many diseases (9). This is said to be due to the presence of various phytochemicals, vitamins and mineral elements which they contain and these attributes have been reported to confer on the plant, astringent, analgesic, anti-helminthic, anti-bacterial, antioxidant, hypoglycemic, antifungal, antisickling, nephroprotective, and other pharmacological properties (8). Phytochemical screening of different parts of unripe *C. papaya* revealed the presence of bioactive compounds such as alkaloids and flavonoids, among others (10).

The complications of SCD are life-threatening and a lot of efforts have been made to manage the disease, especially through the use of drugs that will prevent sickling of red cells that are usually the cause of crises. However, the cost implications and possible toxicity of these drugs constitute a major hindrance to SCD management (11). Therefore, potential management of SCD with a sweet, edible fruit like pawpaw offers a potential means of avoiding the use of drugs with its attendant complications. *C. papaya* is a common plant growing naturally in the community of Ilishan-Remo, Ogun State. There is a dearth of investigation comparing the antisickling activity of *C. papaya* at different ripening stages.

This study aimed to investigate the antisickling and sickling-reversal activities of aqueous extracts and ethyl acetate fractions of unripe, partly ripe, and fully ripe *C. papaya* fruit pulp (CPFP).

**Methods**

Ten sickle cell patients who have not had any crises in the last 72 hours were recruited, from the Haematology Unit of Babcock University Teaching Hospital for the study. The research protocol was carefully explained to the participants after which they signed a written consent form to indicate their interest in participating. The research was carried out between March and October 2019 at the Department of Biochemistry, Babcock University, Ilishan-Remo, Ogun State, Nigeria. Babcock University is located at Ilishan-Remo, equidistant between Ibadan and Lagos. The department of Biochemistry ventures into research in the areas of Phytomedicine, Clinical Biochemistry, Toxicology, Molecular Biology, Biotechnology, and Human Nutritional Biochemistry.

Following authentication, fresh *C. papaya* fruits were collected directly from the *C. papaya* tree at the specific stages of ripening; unripe, partly ripe, and fully ripe. The unripe fruit was defined by the completely green color of the exocarp and the white color of the flesh. The partly ripe fruit was defined by the partly green and partly yellowish color of the exocarp and the light yellow color of the flesh. The fully ripe fruit was defined by the completely yellow color of the exocarp and the orange-red color of the flesh (12). Subsequently, the fruits were peeled, the seeds inside were removed and the fruit pulps were washed with distilled water and kept in a refrigerator, in preparation for analyses.

*Preparation of Aqueous Extracts and Ethyl Acetate Fractions of C. papaya Fruit Pulp*

One hundred grams each of unripe, partly ripe, and fully ripe C. papaya fruit pulp were diced into separate beakers and 100 ml of distilled water was added to each beaker to soak its content at room temperature for 48 hours. The content of each beaker was then filtered using a filter paper (Whatman No. 1, 125mm, England) and the filtrates were collected as the aqueous extract of unripe, partly ripe, and fully ripe *C. papaya* fruit.

Similarly, 100 g each of unripe, partly ripe and fully ripe C. papaya fruit were diced into different beakers, soaked with 100 ml of methanol (BDH Chemicals, Bois-Franc, Canada) and kept at room temperature for 48 hours. The content of each beaker was concentrated to dryness using a rotary evaporator (RE-1050, Shanghai Yuhua Instrument Equipment CO., China) to obtain the crude methanol extract (13). The crude methanol extract was then reconstituted in distilled water and partitioned with ethyl acetate (BDH Chemicals, Bois-Franc, Canada) to obtain the ethyl acetate fraction of unripe, partly ripe and fully ripe *C. papaya* fruits.

*Blood Sample Collection and Processing*

Ten milliliters of blood was collected from each participant by venipuncture, following the method

described by Whitehead (14), and transferred immediately into EDTA bottles. Each sample was subjected to antisickling and sickling-reversal tests within 48 hours of collection. The blood samples were stored at 4 – 8 $^0$C during the period of study. A control experiment was set-up which contained phosphate buffer in place of the extracts and fractions.

*Quantitative Phytochemical Analysis*

The unripe, partly ripe, and fully ripe C. papaya fruit pulps were prepared for phytochemical screening using the method of Oloyede (15). The peeled fruits were chopped and oven-dried at 40$^0$C and then ground with an electric grinder. The resulting powdered samples were stored in properly sealed bottles at 4$^0$C in a refrigerator. These were quantitatively analyzed for alkaloid, saponin, tannin, total phenol, and flavonoid contents.

*Determination of Alkaloid Content*

The alkaloid content was estimated using the method described by Harborne (16). Forty milliliters of 20% acetic acid in ethanol was added to 5 g of the powdered sample, covered and allowed to stand for 4 hours and subsequently subjected to filtration with a filter paper. The filtrate was then concentrated on a water bath to 10ml. Concentrated ammonium hydroxide (BDH Chemicals, Bois-Franc, Canada) was added dropwise to the filtrate until the precipitation was complete. The whole suspension was then allowed to settle and the resulting precipitate was washed with dilute ammonium hydroxide (Sigma Aldrich Chemicals Company, Missouri, United States) and filtered. The residue was dried in the oven (Uniscope SM9053 Oven, Surgifield Medicals, Oakhampton UK) at 60$^0$C and weighed. Percentage composition of alkaloid was calculated as follows:

$$\% \text{ Alkaloid Composition} = \frac{\text{(Final weight – Initial weight)}}{\text{Initial weight}} \times 100$$

*Determination of Saponin Content*

Saponin content was determined using the method of Obadoni and Ochuko (17). Twenty grams of the powdered sample was put into a conical flask containing 100 ml of 20% aqueous ethanol. The sample was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% ethanol (BDH Chemicals, Bois-Franc, Canada). The combined extract was reduced to 20 ml over a water bath at 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added (to remove the fat) and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. This purification process was repeated thrice. Sixty milliliters of n-butanol (BDH Chemicals, Bois-Franc, Canada) were then added. These combined n-butanol-aqueous extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the sample was dried in the oven at 40$^0$C to a constant weight and the percentage Saponin content was calculated as follows:

$$\% \text{ Saponin content} = \frac{\text{(Final weight – Initial weight)}}{\text{Initial weight}} \times 100$$

*Determination of Tannin content*

This was done using Vanillin-HCL method (16) in which 0.2 g of powdered sample was extracted with 10 ml of methanol at 30$^0$C. One milliliter of the resulting extract was reacted with 5 ml of acidified vanillin reagent (50:50 mixtures of 1% vanillin and 8% HCl in methanol) at 30$^0$C for 20 minutes after which absorbance was read at 500 nm. For blank, 4% HCl was added to the extract instead of vanillin reagent, and absorbance was also read at 500 nm. Blank value was subtracted from experimental values to give adjusted data. Catechin was used as standard, and a catechin standard curve from 0.0 to 1.0 mg/ml in 0.2 mg increment was used to calculate tannin levels. The tannic acid content was expressed in terms of milligram catechin equivalent per 100-gram dry weight of the sample.

*Estimation of Total Phenol*

The total phenol content in different solvent extracts was determined using a procedure reported by Singleton, Orthofer, and Lamuela-Raventos (18). Appropriate dilutions of the extracts were mixed with 2.5 ml of 10% Folin–Ciocalteau's reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45$^0$C and the absorbance was measured at 765 nm. The total phenol content was subsequently calculated using gallic acid as standard expressed in terms of milligram gallic acid equivalent per 100 grams' dry weight of the sample.

*Determination of Flavonoid content*

The amount of total flavonoid content was determined by the Aluminum chloride method (19). The reaction mixture (3.0 ml) comprised 1.0 ml of extract, 0.5 ml of aluminum chloride (1.2%)

and 0.5 ml of potassium acetate (120 mm) was and 1 ml of methanol. This was incubated at room temperature for 30 minutes and the absorbance was measured at 415 nm. Quercetin (Sigma Aldrich Chemicals Company, Missouri, USA) was used as a positive control. The flavonoid content was expressed in terms of quercetin equivalent (mg/g of the extracted compound).

*Antisickling Activity*

The antisickling effect of the fruits was determined by the method of Adejumo et al (20). HbSS blood samples (0.2 ml) was pipetted into six test tubes followed by addition of 0.2 ml of the aqueous extracts and ethyl acetate fractions of unripe, partly ripe and fully ripe fruits were placed into six test tubes separately. The mixture was overlaid with 1ml liquid paraffin (to exclude oxygen from the mixture) and incubated in a water bath at 37ºC for 4 hours. Then 0.6 ml of freshly prepared 2% sodium metabisulphite (LOBA Chemie Pvt. Ltd., Mumbai, India) solution was added carefully under the liquid paraffin to the mixture using a syringe and needle. The solution was then mixed and incubated further for another 90 minutes at 37ºC in a water bath. The experiment was set up in triplicates with a negative control test tube containing 0.2 ml phosphate-buffered solution (0.005M, pH 8.0) in place of the extract. After 90 minutes of incubation, liquid paraffin was carefully removed and the resultant mixture was fixed in 3 ml of 5% buffered formalin and kept at ambient temperature until ready for counting. Slides were prepared by placing a drop of the mixture on a clean glass slide and covering with a slip. The edges were sealed with vaseline after gentle pressing to remove the excess mixture. Prepared slides were incubated at $37^0$C for 30 minutes and viewed under a microscope. The percentage antisickling activity of each extract was calculated using the formula:

$$\text{(\%) Antisickling activity} = \frac{\text{Number of non-sickled cells}}{\text{Total number of cells}} \times 100$$

*Sickling-reversal Activity of the Extract*

The sickle-cell reversal effect of the extracts was determined using the method described by Ogunyemi et al (21). 0.2ml of HbSS blood sample was pipetted into six test tubes, and 0.2 ml phosphate-buffered solution (0.005M, pH 8.0) was added to each, the mixture was then covered with 1 ml liquid paraffin. 0.6 ml of 2% sodium metabisulphite solution was carefully introduced into the mixture under the liquid paraffin. The solution was mixed gently and then incubated at 37ºC in a water bath for 90 minutes. At the end of the incubation period, 0.2 ml each of the aqueous extracts and ethyl acetate fractions of the fruits were added to each tube under the liquid paraffin and incubated for another four hours. The experiment was set up in triplicates with a negative control test tube containing 0.2 ml phosphate buffer solution in place of the extract. The liquid paraffin layer was carefully removed using a Pasteur pipette and 3 ml of 5% buffered formalin solution was added at the end of the 4-hour incubation. The solution was thoroughly mixed and kept at ambient temperature until ready for microscopic analysis. Slides were prepared by placing a drop of the solution on a clean glass slide and covering with a slip. The edges were sealed with Vaseline after gentle pressing to remove the excess mixture. The prepared slide was then incubated at $37^0$C for 30 minutes and viewed under a microscope. The percentage sickle cell reversal activity of each extract was calculated using the formula:

$$\text{(\%) Sickling-reversal activity} = \frac{\text{Number of non-sickled cells}}{\text{Total number of cells}} \times 100$$

## Results

The phytochemical contents of *C. papaya* fruits at various stages of ripening are shown in Table 1. The percentage alkaloid composition of the partly ripe *C. papaya* fruit (14.24 ± 3.49%) was significantly lower ($p < 0.05$) than that of the unripe (18.17 ± 0.52%) and fully ripe (19.29 ± 2.50%) fruits. For tannin composition, there was a significant difference ($p < 0.05$) between the three fruits, with the fully ripe *C. papaya* fruit (22.26 ± 0.43 mg CE/ 100 g) having the highest tannin content, followed by the partly ripe fruit (4.84 ± 1.09 mg CE/ 100 g) and the unripe fruit (1.25 ± 0.66 mg CE/ 100 g) having the least. The total phenol composition for the fruits was found to be lower in the unripe fruit (334.12 ± 12.03 mg GAE/100 g) as compared to the partly ripe (450.90 ± 13.83 mg GAE/100 g) and fully ripe (448.29 ± 18.61 mg GAE/100 g) fruits. There was no significant difference ($p > 0.05$) in the flavonoid and saponin concentrations of the fruit at different ripening stages.

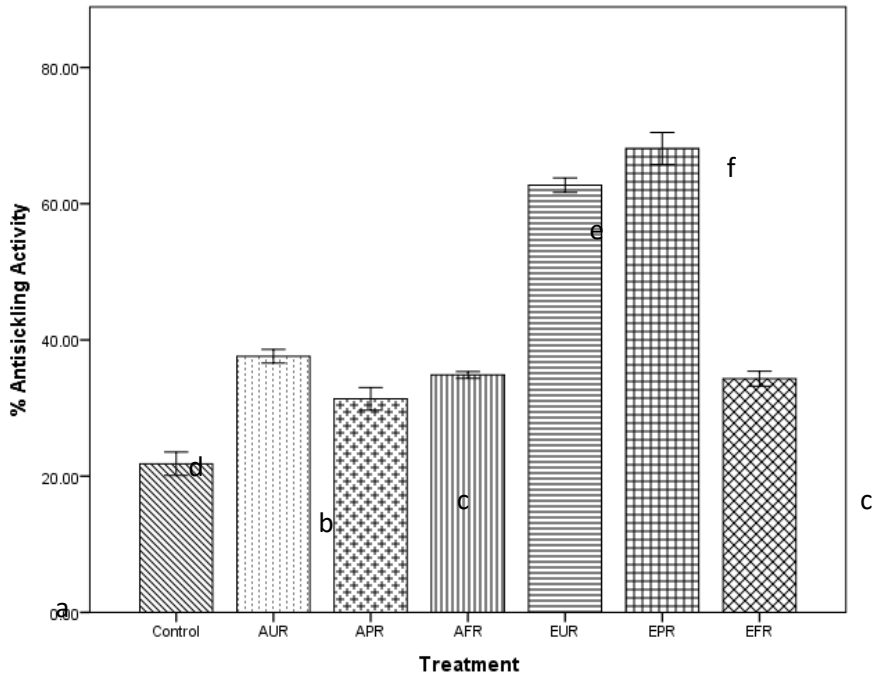
**Table 1: Phytochemical composition of unripe, partly ripe and fully ripe *C. papaya* fruit**

| Phytochemical | *C. papaya* fruit | | |
|---|---|---|---|
| | Unripe | Partly Ripe | Fully Ripe |
| Alkaloid (%) | 18.17 ± 0.52[b] | 14.24 ± 3.49[a] | 19.29 ± 2.50[b] |
| Saponin (%) | 1.58 ± 0.23[a] | 1.66 ± 0.37[a] | 1.82 ± 0.10[a] |
| Tannin (mg CE/ 100 g) | 1.25 ± 0.66[a] | 4.84 ± 1.09[b] | 22.26 ± 0.43[c] |
| Total Phenol (mg GAE/100 g) | 334.12 ± 12.03[a] | 450.90 ± 13.83[b] | 448.29 ± 18.61[b] |
| Flavonoid (mg QE/ 100 g) | 14.69 ± 1.71[a] | 18.14 ± 1.69[a] | 15.87 ± 1.08[a] |

Values are presented as mean ± Standard deviation (SD), n = 3. Values in the same column and having the same superscript letters have no statistically significant difference at $p < 0.05$. CE – Catechin equivalent; GAE – Gallic acid equivalent; QE – Quercitin equivalent

Figure 1. shows the antisickling effect of the aqueous extracts and ethyl acetate fractions of unripe, partly ripe and fully ripe *C. papaya* fruit. The control group had the lowest antisickling activity compared to the treatment groups. The ethyl acetate fractions of the unripe and partly ripe fruits had the highest antisickling effect of 62.72% and 68.11%, respectively. The antisickling effect of the ethyl acetate fraction of the partly ripe fruit was significantly higher ($p = 0.02$) than that of ethyl acetate fraction of the unripe *C. papaya*. In contrast, the aqueous extracts of the partly ripe and fully ripe fruits had the lowest antisickling activity among the treatment groups with no significant difference ($p > 0.05$) between the two fruits.

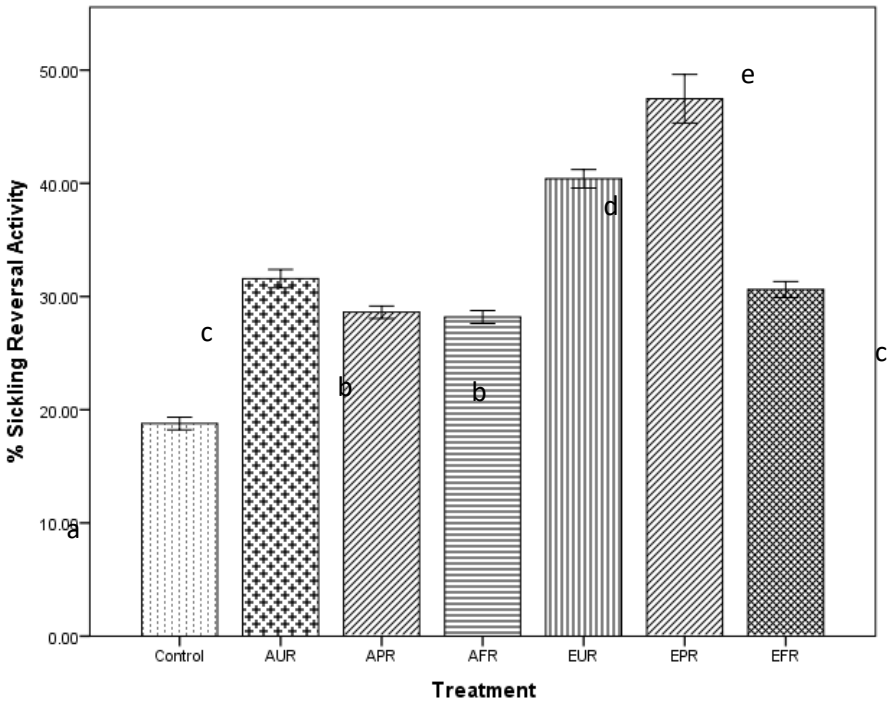
**Figure 1: Antisickling effect of aqueous extracts and ethyl acetate fractions of unripe, partly ripe and fully ripe *C. papaya* fruit**




Bars with the same letters have no statistically significant difference at $p < 0.05$.
AUR – Aqueous extract of unripe *C. papaya;* APR – Aqueous extract of partly ripe *C. papaya;*
AFR – Aqueous extract of fully ripe *C. papaya;* EUR – Ethyl acetate fraction of unripe *C. papaya*
EPR – Ethyl acetate fraction of partly ripe *C. papaya;* EFR – Ethyl acetate fraction of fully ripe *C. papaya*

Figure 2 shows the sickling-reversal activity of the aqueous extracts and ethyl acetate fractions at various ripening stages of the *C. papaya* fruit. All the treatment groups had higher sickling-reversal activities than the control group. The ethyl acetate fraction of the unripe and partly ripe fruits had the highest sickling-reversal activity of 40.40% and 47.47%, respectively. Also, the sickling-reversal activity of ethyl acetate fraction of the partly ripe was significantly higher ($p < 0.05$) compared to the ethyl acetate fraction of the unripe *C. papaya*.

**Figure 2: Sickling-reversal activity of aqueous extracts and ethyl acetate fractions of unripe, partly ripe and fully ripe *C. papaya* fruit**




Bars with the same letters have no statistically significant difference at $p < 0.05$.
AUR – Aqueous extract of unripe *C. papaya;* APR – Aqueous extract of partly ripe *C. papaya;*
AFR – Aqueous extract of fully ripe *C. papaya;* EUR – Ethyl acetate fraction of unripe *C. papaya*
EPR – Ethyl acetate fraction of partly ripe *C. papaya;* EFR – Ethyl acetate fraction of fully ripe *C. papaya*

## Discussion

*C. papaya* fruit, at different ripening stages, contains phytochemicals such as alkaloid, saponin, tannin, phenol, and flavonoids. Previous studies have reported the presence of these phytochemicals in *C. papaya* fruit, stem, leaves, seeds, and bark (22, 23). These compounds have been reported to contain the active principle for plants containing antisickling agents (24).

Ibrahim et al (25) reported that antisickling activity of *Hymenocardia acida* Tul was found in the fractions containing flavonoids and saponin. Furthermore, flavonoids have been shown to protect against hemoglobin oxidation and other cellular modifications promoted by peroxides in SCD (26). Kumar and Pandey (27) reported that the functional hydroxyl groups in flavonoids mediate their antioxidant effects in sickle cells by scavenging free radicals and/or by chelating metal ions.

Hence, the results of this study are in congruence with earlier reports where *Hymenocardia acida* (25), *Plumbago zeylanica* (20), *C. cajan* leaf (10), *Zanthoxylum heitzii roots* (7, 28), and unripe fruit,[29] seeds and leaf extracts (30) of *C. papaya* have been screened for the presence of these phytochemicals. Each phytochemical is of specific importance in its application for the management of SCD in ethnomedicine. Alkaloids have been reported to show stimulatory effects on the nerves and also act as anticonvulsants and muscle relaxants (31). This property of alkaloids may help in the management of pain associated with vaso-occlusive crises in SCD. Saponins, phenols, and tannins have been found to possess antioxidant activities (32) which may help to prevent the generation of free radicals and prevent oxidative damage to cells and tissues in patients with SCD. The presence of these phytochemicals in the extracts and fractions of *C. papaya* fruit probably explains the reason for the antisickling properties elicited.

Oduola *et al* (13) reported that the ethyl acetate fraction of unripe *C. papaya* fruit contains an antisickling agent, which was identified as caricapinoside (8(2-0-β-D-4, 5-anhydroglucitoyl 1→ 2glucopyranosyl carbonyl) dibenzo [b,e] [1,4] dioxine-2-carboxylic acid). Findings from this present study revealed that the unripe, partly ripe, and fully ripe pulp of *C. papaya* have antisickling and sickling-reversal activities but the ethyl acetate fraction of partly ripe *C. papaya*

possessed the highest antisickling activity when compared to other extracts and fractions of the unripe and fully ripe fruits. This finding suggests that the partly ripe fruit may also contain caricapinoside as previously reported for the unripe fruit (12), and perhaps at a higher concentration than the unripe *C. papaya* fruit. This could account for its effectiveness in preventing the sickling of erythrocytes on exposure to a sickling agent (sodium metabisulphite). This may also be the bioactive agent responsible for the sickling-reversal activity shown by the ethyl acetate fraction of the partly ripe *C. papaya* fruit which was significantly higher than that of the unripe fruit.

Findings from this study justify the use of *C.* papaya for the management of SCD by traditional healers. It may also route to the discovery of bioactive compounds that may be used in the development of drugs in the management of SCD, by medical practitioners which will be affordable and have very minimal or no side effects. However, this study is a preliminary investigation from which further studies will be designed to clarify the findings.

## Conclusion

*C. papaya* fruit, at different stages of ripening, contains important phytochemicals at varying concentrations. Aqueous extracts and ethyl acetate fractions of *C. papaya* fruit showed antisickling and sickling-reversal activities. These activities may synergistically help to reduce the associated complications of sickle cell disease when consumed by affected individuals.

It is recommended that further research should be conducted to determine the presence and composition of caricapinoside in *C. papaya* fruit at different stages of ripening. Also, the mechanism(s) of action of the aqueous extracts and ethyl acetate fractions of *C. papaya* fruit at different stages of ripening should be investigated.

## Declarations

*Ethics approval and consent to participate*

Ethics Approval for the study was obtained from the Babcock University Health Research Ethics Committee with reference number: BUHREC104/19.

*Consent for publication*

The authors hereby give consent for the publication of our work under the creative commons CC Attribution-Noncommercial 4.0 license.

*Availability of data and materials*

The data and materials associated with this research will be made available by the corresponding author upon reasonable request.

*Competing interests*

The authors declare no conflict of interest.

*Funding*

The authors did not receive any funding for the research

*Authors' contributions*

All authors contributed equally to the design, methodology, data collection, write-up, and the final editing of the manuscript.

*Acknowledgment*

The authors acknowledge the laboratory technologists who assisted in the conduce=t of the research.

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