

Antisickling Properties of *Carica papaya* Linn.

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(Received 29 May 2008; Revised 07 June 2008; Accepted 14 June 2008)

ABSTRACT

Present study deals with the antisickling properties of *Carica papaya* (Family-Caricaceae) fruit pulp in distilled water, methanol and chloroform using sodium metabisulphite sickled red blood cells. The highest antisickling potencies of 87% inhibitory and 74% reversal activities were obtained from the 5-day fermentation products at the optimum concentration of 2.5mg/ml. The methanol extract gave 64% inhibitory and 55% reversal activities while the chloroform extract was inactive. The amino acids, phenylalanine, tyrosine and glycine already reported in the unripe fruit of *Carica papaya*, which are the possible antisickling components and responsible for their antisickling activity.

Keywords: Antisickling; Aqueous and solvent extracts; *Carica papaya*.

INTRODUCTION

Sickle cell anaemia is an inherited chronic disease in which the red blood cells (RBC) become crescent-shaped instead of disc-shaped. It is a genetic disease caused by abnormal haemoglobin called sickle haemoglobin (HbS), which polymerizes under deoxygenated condition and deforms the red blood cells into a 'sickle' shape (Pauling, et al., 1949). Nucleotide sequencing of β -globin mRNA from sickle β -globin genes revealed that the normal codon GAG at position β^6 has been replaced by GUG (Marotta and Wilson, 1977). This change determines the insertion of valine at this position instead of the

glutamic acid that occurs in HbA (Perutz and Lehmann, 1968). The disease and trait occur in people of African descent, Mediterranean countries, India and the Middle-East but rarely in people of European and white decent.

Over the years, a variety of chemical agents have been proposed for reversing the sickle shape of erythrocytes *in vitro*, however the first plant extract reported to have reversed sickled cells *in vitro* was the aqueous extract of the plant *Zanthoxylum xanthoxyloides* Waterm (Rutaceae) (Sofowora and Isaac-Sodeye, 1971). First time Thomas and Ajani (1987) reported antisickling properties of *Carica papaya* and suggest that active compound(s), preventing and reversing sickling could be organic acids, produced after hydrolysis of corresponding esters in the fruit.

Carica papaya Linn. (Family Caricaceae) is a perennial, herbaceous plant, with copious milky latex reaching to 6-10 meters in height, the stem up to 30 cm thick, simple or branched above the middle and roughened with leaf scars. The unripe fruit is used traditionally among the Yoruba tribe of Nigeria for treating jaundice and for the management of sickle cell anaemia (Elujoba, 2001: Personal communications). Their unripe fruit contains glycine, phenylalanine, and tryptophan with reported antisickling properties. (Pizzorno, et al, 1985, Igbal and Kazi, 1980).

MATERIALS AND METHODS

Plant Material: The unripe fruit of *Carica papaya* Linn (Caricaceae) was collected from the premises of the College of Health Sciences, Obafemi Awolowo University Ile-Ife, Nigeria and authenticated at the Botany Department herbarium of the same institution.

Other materials: Two percent (2%) w/v sodium metabisulphite solution, phosphate buffered saline solution, 5% buffered formalin solution, vanillic acid, para-hydroxy benzoic acid (PHBA) and Siculine Syrup^{TR}

Fermentation of fresh unripe *Carica papaya* fruit: The matured, unripe, fresh fruit of *Carica papaya* (collected close to the topmost of the tree) was collected from the tree. The greenish epicarp was peeled off, the whitish seeds discarded while the pulp was cut into small pieces, 180g of which was accurately weighed into each of the 16 conical flasks in 8 pairs of duplicates and 300ml distilled water added to each flask. These were kept at room temperature for 24 to 168 hours. An extract tagged P0 was prepared from a pair on day 0 by blending the fruit pulp and extracting immediately. Two flasks were thereafter removed every 24 hours, filtered and the residue discarded while the filtrates were concentrated by boiling to give aqueous extracts which were allowed to cool and kept at 4°C until ready for use. The aqueous extracts were tagged P1, P2, P3, P4, P5, P6, and P7 representing 24,48,72,96,120,144 and 168-hour fermentation, respectively.

Fractionation of *Carica papaya* extracts: The extracts of the fermented *Carica papaya* fresh unripe fruit with the highest antisickling activity was further purified by partitioning with butanol and ethyl acetate successively giving three fractions viz: aqueous, butanol and ethyl acetate. The butanol and ethyl acetate fractions were evaporated to dryness *in vacuo* and then re-dissolved in water. The fractions were then used for the antisickling experiments.

Preparation of Methanol and Chloroform extracts

13g dried powdered *Carica papaya* was macerated in 300ml methanol and 300ml chloroform separately for 48hours at room temperature. Each was filtered afterwards and the extract evaporated to dryness *in vacuo* by using the rotary evaporator. The resulting dried extracts were then re-dissolved in water and used for the antisickling tests.

The Antisickling Assay:

(i) Collection of Blood Samples: 5ml of fresh whole blood sample were drawn, by venipuncture, from each sickle cell anemia patient in steady state between the ages of 12 and 23 years (both sexes) into an EDTA (Ethylene Diamino Tetra-acetic Acid) bottle using a new set of disposable syringe and needle for each patient. Blood samples were collected every week, only from confirmed sickle cell patients attending the regular haematology outpatient clinic at the Obafemi Awolowo University Teaching Hospitals Complex* (OAUTHC). (**Note:** There was permission already for Sickle cell anaemia research at the teaching hospital. I was therefore permitted by the haematology department to use part of the blood collected for their other ungoing researches). The blood was mixed carefully but properly to avoid clotting and fresh blood samples were always used within the first 48hours of collection.

(ii) Inhibitory Antisickling Test: 0.2ml of homozygous sickle cell anaemia whole blood was placed in a test tube (in duplicates). Then, 0.2ml phosphate buffered solution plus 0.2ml of the aqueous extract of fermented fresh *Carica papaya* fruit were added and the mixture immediately covered with 1ml liquid paraffin and incubated in a thermostated water bath at 37°C for four hours. After the 4-hour incubation period, 0.6ml of freshly prepared 2% sodium metabisulphite solution was carefully added under the liquid paraffin to the incubation mixture and the final mixture was thoroughly but carefully mixed by rolling the test tube between the palms. The mixture was incubated for another 1½ hours at 37°C in the water bath. At the end of the incubation period, the liquid paraffin was carefully removed by the use of a Pasteur pipette and the remaining mixture was fixed in 3ml of 5% buffered formalin solution and reserved until ready for counting.

The control experiments consisted of 0.2ml phosphate buffered saline solution to replace the extract (negative control), 0.2ml vanillic acid solution and 0.2ml Siculine syrup (a traditional antisickling herbal remedy) to replace the extract, representing the positive controls. The percentage inhibitory activity for each sample was then calculated from the results and presented as duplicate means for all the samples, including the negative and positive experimental controls. These procedures were repeated for the chloroform and methanol extracts of the dried *Carica papaya* powdered fruit and also the partitioned fractions of the most active aqueous extract.

(iii) Reversal Antisickling Test: 0.2ml HbSS whole blood was placed in a test tube (in duplicate), 0.2 ml phosphate buffered solution was added and the mixture covered with 1ml liquid paraffin. 0.6ml 2% sodium metabisulphite solution was introduced under the liquid paraffin into the blood layer. The mixture was thoroughly but carefully mixed by rolling

the test tube between the palms before incubating at 37°C in a thermostated water bath for 1½ hours. At the end of the incubation period, 0.2ml of the aqueous extract of the fermented unripe *Carica papaya* fruit was added under the liquid paraffin carefully as before and incubated for another 6 hours. The experiment was set up in duplicates with a negative control where 0.2 ml phosphate buffered saline solution was used in place of the extracts while 0.2ml p-hydroxy benzoic acid solution and 0.2ml Siculine syrup were used separately as positive controls.

At the end of the six-hour incubation, the liquid paraffin layer was carefully removed by the use of Pasteur pipette and 3ml of 5% buffered formalin solution was added. The mixture was thoroughly mixed to ensure proper fixation and kept until ready for counting. The percentage reversal activity for each sample was then calculated and presented as duplicate means of all the samples including the experimental controls. These procedures were repeated for the chloroform and methanol extracts of dried *Carica papaya* and also the partitioned fractions of the most active aqueous extract.

(iv) Counting of treated blood cells: The fixed cell mixtures were each centrifuged and the supernatant decanted. With a capillary tube, one or two drops were applied on a microscope slide, carefully covered with a cover slip and with a high power microscope objective (x100), 400 cells (both sickled and unsickled erythrocytes) were counted and the percentage sickled cells calculated there from.

Determination of the concentration of Carica papaya extract in the incubation mixture:

(i) Fermented fresh Carica papaya fruit extract: Fresh unripe *Carica papaya* fruit (180g), in triplicate was fermented in 300ml distilled water at room temperature for 5 days. After the incubation period, the mixture was filtered giving 330 ml as the filtrate (there was an increase in volume because of the water present in the fresh fruit fermented), which was boiled while the marc was discarded. The extract was freeze-dried and the resulting dried extract was accurately weighed. The weight of *Carica papaya* fruit, equivalent to the 0.2ml of the extract used for the antisickling assay was then calculated as follows: The 330ml aqueous extract obtained by incubating 180g of fresh pawpaw for 5 days filtered and the filtrate freeze dried at 4°C, gave the mean value of 5.25g dry extract.

Therefore 330ml aqueous extract of fresh pawpaw gave 5.25g dried extract so 0.2ml of the aqueous extract will be equivalent to 0.0032g dried *Carica papaya* extract.

(ii) Fermented powdered Carica papaya fruit extract: Powdered *Carica papaya* fruit (15g) was accurately weighed into conical flasks in triplicate and fermented in 300ml distilled water at room temperature for five days. After the incubation period, the mixture was filtered and the filtrate (210ml) boiled while the marc was discarded. The extract obtained, tagged PP5, was then freeze dried and the resulting dried extract was accurately weighed. The weight of *Carica papaya* powdered fruit equivalent to the 0.2ml of the extract used for the antisickling assay was then calculated as above as follows: The extract obtained (210ml) by fermenting 15g dried powdered *Carica papaya* fruit for five days, in 300ml distilled water, filtered and the filtrate freeze dried, gave the mean value of 3.40g dry extract

Therefore, 210ml gave the mean value of 3.40g dried extract then 0.2ml will be equivalent to 0.0032g dried extract.

(iii) *Varying the concentration of fermented powdered Carica papaya fruit pulp:* Fermented powdered *Carica papaya* dried fruit extract was prepared same as (ii). The volumes of the extract used for the antisickling tests were then serially varied from 0.1ml to 0.5 ml in order to investigate the effect of concentrations of the extracts on antisickling potencies of the samples. The procedures were investigated for both the inhibitory and reversal activities.

RESULTS

Fermented fresh Carica papaya fruit: The fresh unripe *Carica papaya* fruit, fermented at room temperature for five days (P5) gave the lowest percentage (%) sickled cells and hence the highest inhibitory and reversal activities (Figures 1 and 2). The photomicrographs showing the results of the control experiments as well as the inhibitory and reversal activities are shown in Plates 1, 2 and 3.

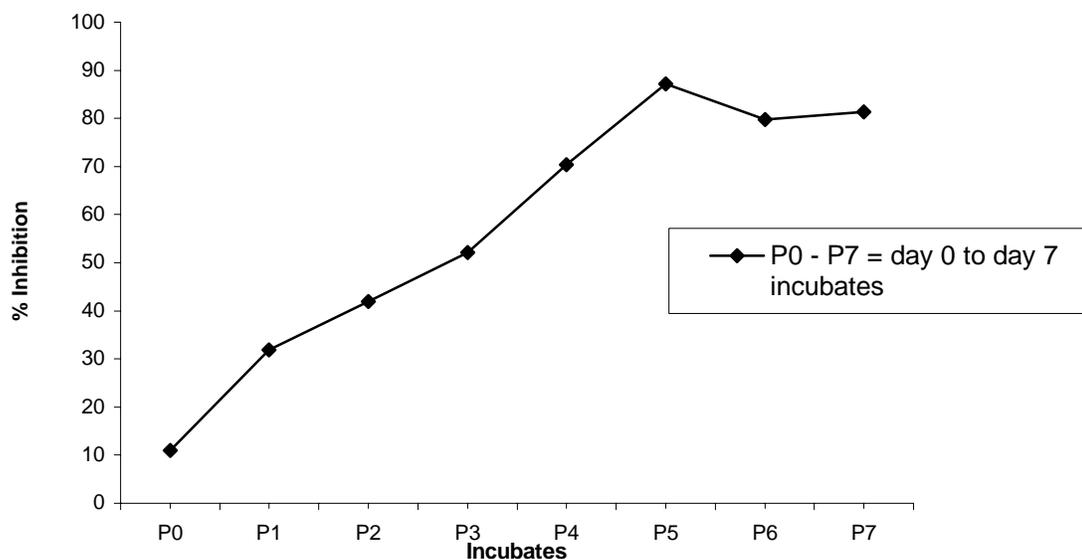


Figure 1: % Inhibitory activities of fermented fresh *Carica papaya* pulp.

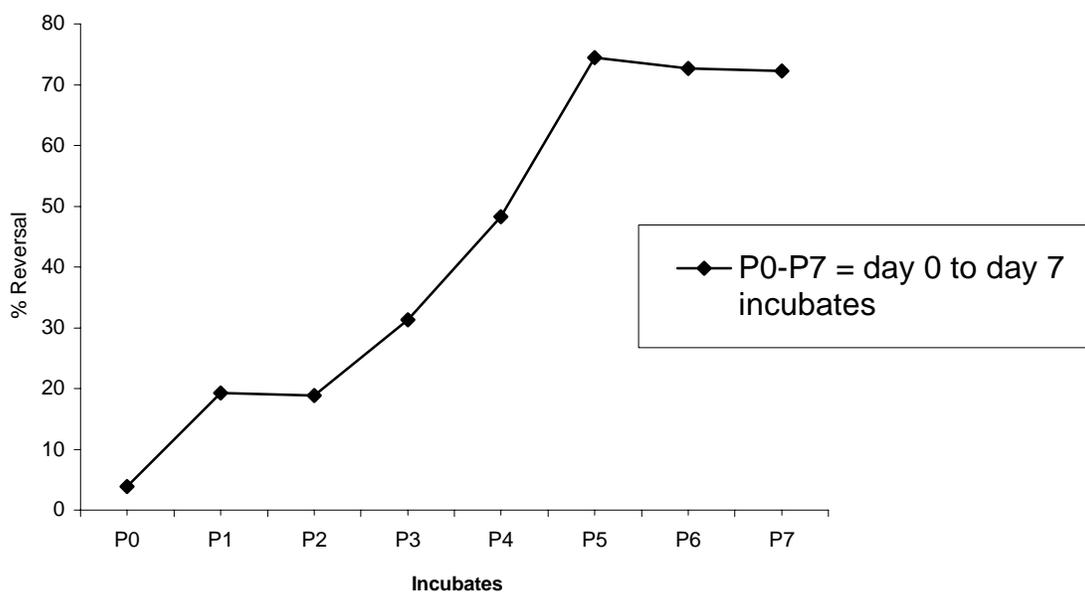


Figure 2: % Reversal activities of fermented fresh *Carica papaya* pulp

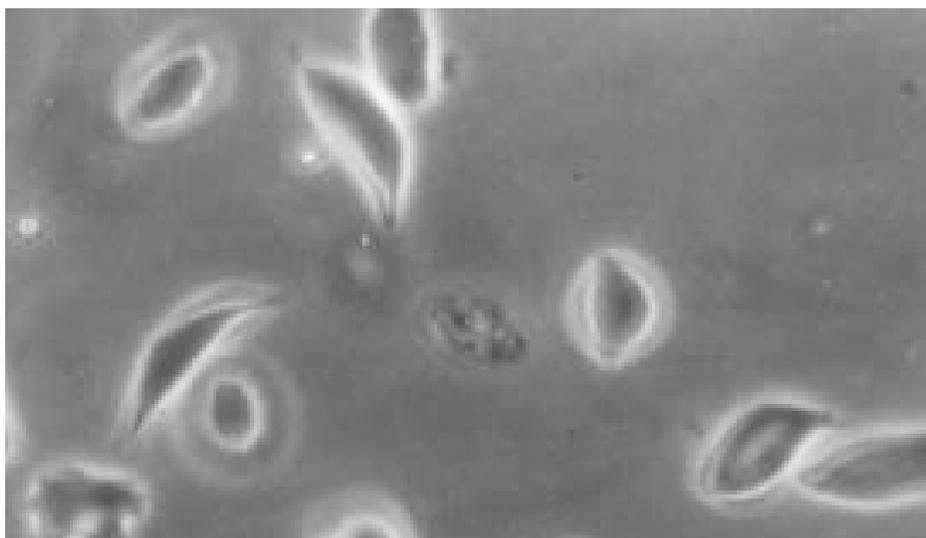


Plate 1: Untreated Control showing irreversibly sickled cells.



Plate 2: Inhibitory activity of 5-day fermented fresh *C. papaya* fruit pulp (87% Inhibition).

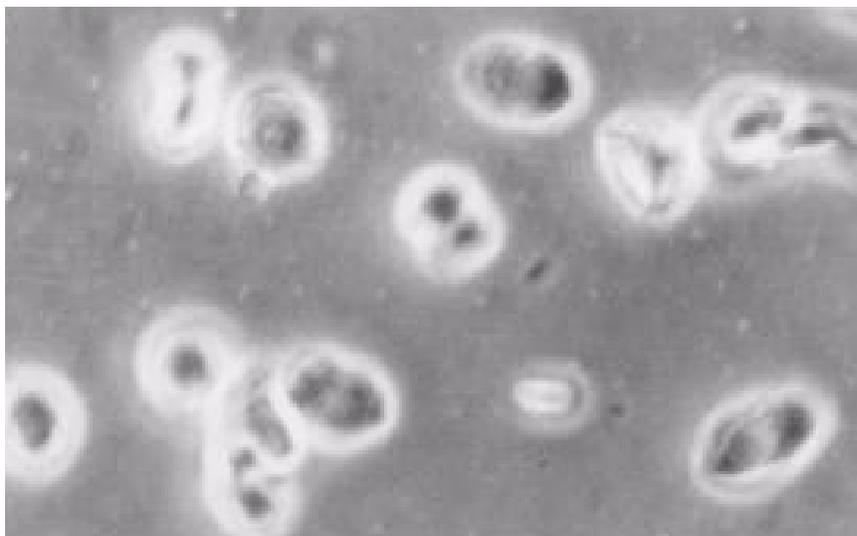


Plate 3: Reversal activity of 5-day fermented fresh *C. papaya* fruit pulp (74% Reversal).

Varying the concentration of fermented *Carica papaya* dried fruit: The results of the inhibitory and reversal activities of different concentrations of *Carica papaya* fruit extract are shown in figures 3 and 4. While the inhibitory and reversal activities of the different fractions from the partitioned pawpaw extract are shown in table 1 and 2.

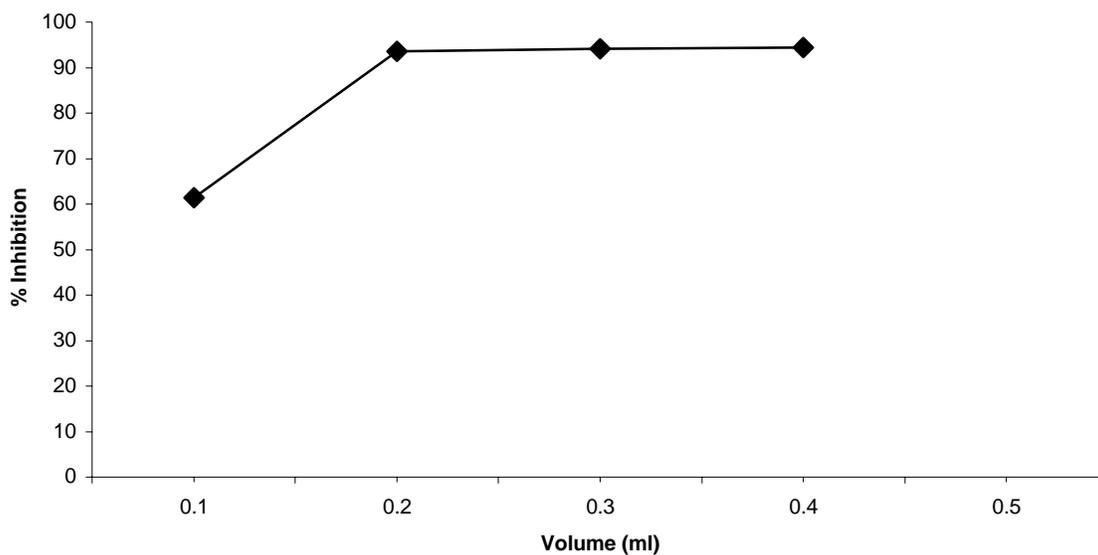


Figure 3: % Inhibitory activities of varying concentrations of fermented dried *Carica papaya* pulp at day 5 incubation (PP5)

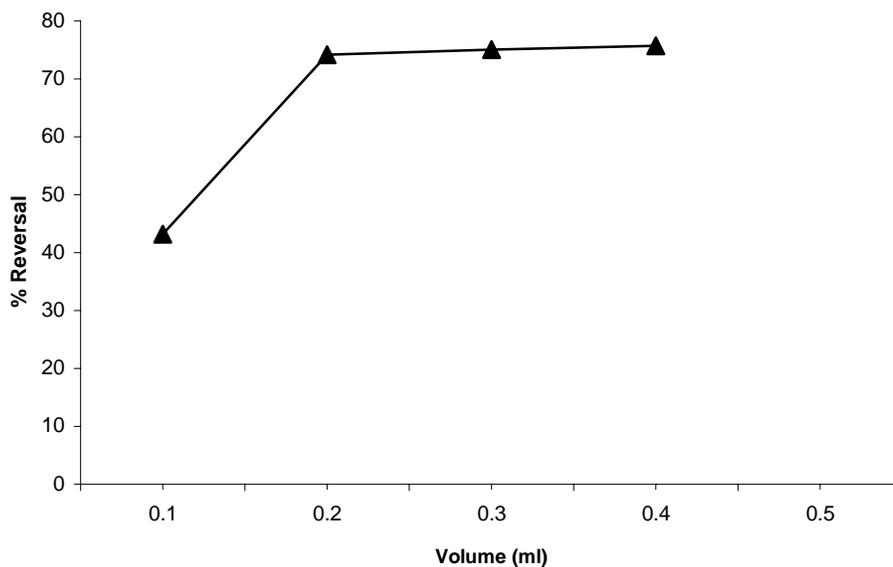


Figure 4: % Reversal activities of varying concentrations of fermented dried *Carica papaya* pulp at day 5 incubation (PP5)

Table 1: Inhibitory and reversal antisickling activities of different fractions from the partitioned *Carica papaya* extract.

Fractions of fermented <i>C. papaya</i>	% Antisickling activities	
	Inhibition	Reversal
Aqueous	93	87
Butanol	21	16
Ethyl Acetate	6	2
Phosphate Buffered Saline	0	0
Vanillic acid	58	-
p-hydroxy benzoic acid	-	52

Table 2: Inhibitory and Reversal activities of methanolic and chloroform extracts of *Carica papaya*.

Extractives	% Inhibition	% Reversal
Methanol	64	55
Chloroform	15	14

DISCUSSION

The antisickling activities of fermented *Carica papaya* fruit pulp could be due to the fact that the active component(s) responsible for the activities was released into the medium at the highest concentration on day 5 of fermentation (Figures 1 and 2). Antisickling activity appeared to have increased with incubation period. The fermented *C. papaya* produced higher inhibitory activity (93%) than vanillic acid and higher reversal activity (87%) than p-hydroxy benzoic acid (Table 1).

From the experiment involving freeze-drying of the fermented *C. papaya* extracts, 0.2ml extract used for the experiment was equivalent to 3mg *C. papaya*, hence the total volume of the incubate, 1.2 ml consisting of 0.2ml PBS, 0.2ml blood, 0.6ml sodium metabisulphite and 0.2ml extract, contained 3mg *C. papaya*, calculating as 2.5mg/ml. The experiment involving different concentrations of the fermented *C. papaya* extract used in the antisickling tests showed that 2.5mg/ml was the optimum concentration as there was no significant difference in the activities given by the higher concentrations (Figures 3 and 4). The 0.5ml volume (6.25mg/ml) caused the red cells to clump together suggesting that the concentration was too high for the blood cells.

The result of the antisickling tests on the partitioned fractions from the fermented products (Table 1) showed that the antisickling agent(s) in *C. papaya* is a relatively polar substance as shown by the result of the aqueous fraction. The aqueous fraction gave 93% inhibitory and 87% reversal antisickling activities, respectively. The butanol and ethyl acetate fractions gave only 21% and 6% for inhibitory but 16% and 2% for reversal antisickling activities, respectively. This result shows that the active antisickling compound is mostly soluble in water. The result of the inhibitory and reversal antisickling test for the methanol extract of *Carica papaya* gave 64% and 55% respectively while that of

chloroform extract showed no positive activity for both inhibitory and reversal test (Table 2). This can further confirm that the antisickling agent in *Carica papaya* is a relatively polar substance. Thomas and Ajani (1987), suggested that the active compound(s) in the soaked or fermented unripe *C. papaya* fruit, preventing and reversing sickling, could be organic acids, produced after the hydrolysis of corresponding esters in the fruit.

Noguchi (1977) has reported the effect of amino acids on gelation kinetics and the solubility of sickled cells while Igbal and Kazi (1980) observed that the aromatic amino acids, phenylalanine, tyrosine and tryptophan were significantly more active as antisickling agents than other amino acids. Duke (1992a) has suggested that the amino acids, glycine and phenylalanine, were the antisickling agents in *C. papaya* fruit while Ekeke and Shode (1990) reported earlier that phenylalanine was the most prominent antisickling constituent of *Cajanus cajan* seeds. The three aromatic amino acids already reported by Igbal and Kazi (1980): tyrosine, phenylalanine and tryptophan, are present in *C. papaya* unripe fruit (Duke 1992a). It could therefore be suggested that these amino acids might be part of the antisickling components of the unripe fruit of *C. papaya*. Furthermore, Ohnishi and Ohnishi (2001) studied the *in vitro* mechanism of dense cell formation caused by deoxy-oxy cycling and found that nutritional antioxidants could inhibit the formation of dense cells when they are employed in the combination of vitamin C, vitamin E and aged garlic extracts. Many constituents of *Carica papaya* fruit including vitamin C, beta-carotene, citric acid, gamma-terpinene, lycopene, methionine, alanine, sucrose and tartaric acid have been reported to have antioxidant activities (Duke 1992b) which could also contribute to its observed antisickling properties.

CONCLUSION

It is therefore proposed that *C. papaya* could serve as a suitable nutritional supplement as well as providing antioxidant therapy necessary for the prevention of dense cell formation and free radical-mediated oxidative cell injury leading to the prolongation of red cell life.

Acknowledgement: I wish to acknowledge the staff of the Department of hematology, Obafemi Awolowo University, Ile-Ife, Osun State Nigeria for the role they played in the success of this research.

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