World Journal of Medical Sciences 5 (4): 98-104, 2010
ISSN 1817-3055
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Some Fruit Juices as Environmental Sickling Agents

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Abstract: Interest in cheap, safe and effective management of sickle cell disease (SCD), an oxidant stress-loaded problem is growing. This *in vitro* study focuses on the effect of some fruit juices on sickle cell hemoglobin polymerization, lactate dehydrogenase (LDH) activity and Fe^{2+}/ Fe^{3+} ratio of sickle cell blood. Ripe fruit juices were prepared from six commonly consumed fruits namely: Grape (*Citrus paradisi*); Lemon (*Citrus limon);* Sweet orange (*Citrus sinesis*); Pawpaw (*Carica papaya-not a citrus fruit*), Sour lime (*Citrus aurentifolia*) and Mandarin (*Citrus reticulata*). Total vitamin C concentration was estimated and expressed in mg/100 g of sample. *Citrus sinesis* (59.0±0.1), *Carica papaya* (41.52±0.02), *Citrus paradisi* (39.80±0.0), *Citrus limon* (16.20±0.1), *Citrus aurentifolia* (27.36±0.2), *Citrus reticulata* (17.08±0.0) respectively. The pH values of the extracts were estimated at 25°C and expressed as follows: 4.75±0.1, 5.27±0.1, 4.61±0.2, 2.80±0.2, 2.20±0.1 and 5.0±0.0 respectively. Amino acids were quantified for each sample and their total concentrations expressed in mg/100 g: 0.290±0.01, 0.110±0.0, 0.250±0.01, 0.130±0.0, 0.094±0.01and 0.087±0.01 with respect to the order above. Except in lemon, phenylalanine (a known antisickling amino acid) was prominent in all water-soluble (WAS) fractions. We demonstrated that vitamin C and phenylalanine can inhibit polymerization of deoxygenated HbSS by about 80.40% and 95.87% respectively in 30 minutes at 100 µM ascorbic equivalence. Two of the citrus samples-*Citrus sinesis*, *Citrus reticulata* and *Carica papaya* (although not a citrus fruit), all inhibited sickle cell hemoglobin polymerization, while *Citrus paradisi*, *Citrus limon* and *Citrus aurentifolia enhanced polymerization,* increased the Fe^{2+}/Fe^{3+} ratio by about 50% and LDH activity usually raised in SCD was significantly raised by *Citrus limon* (66.60%), *Citrus paradisi* (69.20%) and *Citrus aurentifolia* (79.04 %); while *Citrus sinesis*, *Citrus reticulata* and *Carica papaya* reduced it by 42.90%, 25.79% and 17.80% respectively. These sickling fruit juices and their by-products may pose serious health problems for sickle cell disease patients and may constitute an environmental hazard to patients with similar hematological complications.

Key words: Fruits juices · Sickle cell disease · Hemoglobin polymerization · Fe^{2+}/ Fe^{3+} ratio · Environmental sickling agents

INTRODUCTION

Sickle cell disease results from the substitution of an amino acid residue, valine for glutamic acid at the sixth position in the beta (β) chain of the hemoglobin molecule [1]. With few minor exceptions, people with only one gene for hemoglobin S (HbAS) are phenotypically normal (sickle cell trait). People who inherit two HbS genes from their parents have sickle cell disease (HbSS). The relationship between sickle (SCD) and nutrition had been reviewed [2]. Sickle cell disease remains the one chronic disease in which the role of nutrition has been poorly stressed. Over the years, so many investigations have been carried out on agents that can reduce or increase gelation or polymerization [3]. Many environmental factors have been implicated in sickling such as cold, malarial infestation, typhoid fever, sepsis or bacterial infections resulting in aplastic crisis and anemia [4]. Aplastic crisis is a potentially deadly complication of sickle cell disease that develops when erythrocyte production temporarily drops. Many patients with sickle cell disease are anemic but the degree of anemia varies. Many researchers have reported the effect of diet to overcome sickle cell disease [5-7]. Citrus fruits are known

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for flavor, fragrance and juice-ness. Citrus calcium, copper, potassium, magnesium, niacin and vitamin B_6. Lemon is the most common citrus fruit which is oval or round in fruits also called 'acid fruits' and possess many therapeutic benefits due to their detoxifying nature. Citrus fruits belong to the family of plants called Rutaceae [8]. Citrus fruits are rich in vitamin C. They are good sources of minerals, carbohydrates and fibers and also contain essential nutrients like shape, having an acidic juicy flesh, very much richer in vitamin C, used in soft drinks like lemonade. Grape fruit is rounded in shape having large-yellow acidic fruit with acidic juicy pulp. It can be eaten raw or used in preparing marmalade. Mandarin is a small reddish-orange colored, loose skinned citrus fruit. It is used as a sweetener in many grape fruit juices[9]. Citrus fruits contain some plant secondary metabolites such as flavonoids of which examples include-hesperidin (a glycoside of flavanone), quarcitrin, rutrin and the flavone (tangeritin). It is stipulated that hersperidin and rutin may have beneficial effects on capillary permeability and blood flow. They also exhibit anti-allergy and anti-inflammatory benefits of quarcitrin from *in vitro* studies. Quarcitrin can also inhibit reverse transcriptase, an enzyme which is part of the replication process of retroviruses [10]. The beneficial effects of vitamin C, vitamin E and other foodstuffs like legumes have been documented [6]. It is now pertinent that some fruits which are naturally endowed with vitamins C, A, E and other nutrients can exacerbate some of the pathophysiological complications of the syndrome. There are different types of citrus species which abound globally. For example, sour orange (*Citrus aurentum* or *aurentifolia* or *bergemia*) has been associated with many therapeutic claims. It has a complex chemical make up, but very important is the oil extracted from the peel. It is used as a remedy for a variety of health problems. The peel contains flavones, alkaloids, synerphrine, octapamine, N-methyltyramine and carotenoids. In folkloric medicine, the flowers of bitter orange has also been used in the treatment of gastrointestinal problems, nervousness, insomnia, gout, sore throat and obesity [11]. It has been reported that bitter orange is safe at small doses but the reverse is the case when consumed in large amounts. Due to the presence of synerphrine and N-methyltyramine, it can cause hypertension and cardiovascular toxicity [12]. Large amounts in children can cause convulsions and even result in death. Pregnant and breast feeding mothers should not take the herb [11,12]. It is now obvious that some of the fruit juices and their bye-products could produce life-threatening consequences when consumed by sickle cell disease patients. Some registered fruit juices are preserved with sodium metabisulphite which is a universal sickling or deoxygenating agent. Many parts of citrus fruits have been used as medication over the years. The fruits of grape and sweet orange (*Citrus paradisi* and *Cirus sinesis*) have been used for the treatment of tuberculosis [13]. Considering the enormous benefits accruing to humanity from citrus fruits and juices; we were therefore prompted to channel our energies to investigate the effects of some fruit juices on hemoglobin polymerization, Fe^{2+}/Fe^{3+} ratio and lactate dehydrogenase activity of sickle cell blood to assess their suitability for consumption or otherwise by this group of patients whose system is under constant flux of nutrient degradation.

MATERIALS AND MEHODS

The following species of ripe fruits were purchased from a local market at Owerri, capital city of Imo State in Nigeria. The fruits were authenticated as being of good quality and variety by a Crop Scientist at the department of Crop Science and Technology of the University. The fruits include: Sweet orange (*Citrus sinesis*), Pawpaw (*Carica papaya*-although not a citrus fruit, is used here to show the antisickling effect of other fruit sample), Grape(*Citrus paradisi*), Lemon (*Citrus limon*), Sour lime (*Citrus aurentifolia*) and Mandarin (*Citrus reticulata*). Known weights (1000 g) of each species of the samples were squeezed and the expressed juices decanted by means of a Deluxe Juice Extractor (ST-888J). The squeezed fruit juices were deproteinized with 10 ml of 10% saturated alum solution. The juices were partitioned into water-soluble (WAS) and fat-soluble (FAS) fractions using dichloromethane/methanol. Amino acids were identified by TLC and subsequently quantified with ninhydrin. Vitamin C was estimated by titration with 2,6-dichlorophenolindophenol (DCPIP) [14].

Serum and Blood Sample Collection: 5-10 ml blood was collected by venupuncture from confirmed HbSS patients who routinely attend clinic at the Federal Medical Center, Owerri. The subjects were aged between 3 and 30 years and of both sexes. Blood samples were collected into Lithium sequestrene potassium EDTA tubes, hemolyzed by freeze thawing and centrifuged at 1500Xg for 30 minutes. The supernatant (hemoglobin) was transferred to small vials, capped and kept at 8°C in a refrigerator. Serum samples were also collected with Pasteur pipettes and used for assay within 24 hours.

Determination of Total Vitamin C Concentration: The determination of vitamin C concentration of the WAS phase of the ripe fruit juices was carried out by the methods of [14]. The pH values of the juices were equally determined at 25°C. Ascorbic acid standard was prepared containing 1g/dm^3 of ascorbic acid (vitamin C). A solution (0.01%) of 2, 6-dichlorophenolindophenol was used to fill the burette. The vitamin C concentrations were expressed in mg/100g of sample.

Total Free Amino Acid Concentration: This was carried out with ninhydrin reagent using Phenylalanine (Phe) as standard and reading the developed purple color spectrophotometrically at 570 nm. Thin layer chromatography (TLC) was carried out using silica gel and a solvent system of BuOH: HAc:H_2O (4:1:1), was used in the identification of the major amino acids present in the extracts by using their R_f values against the standards.

Polymerization Inhibition Experiment: Polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm by using 2% solution of sodium metabisulphite [15]. The rate of hemoglobin polymerization was inhibited by the addition of 100µM, final assay concentration of the fruit juices. The rate of polymerization inhibition by the antisickling agent was estimated by calculating the tangent of a plot of change in optical density (ΔOD 700 nm) against time in minutes. The rates were equally expressed as percentage with respect to the control. This gives the relative percent polymerization and hence, the relative percent inhibition.

Determination of the Fe^{2+}/Fe^{3+} Ratio: The Fe^{2+}/ Fe^{3+} ratio was determined by the methods of [16,17]. The approach used employs the established procedure of lysing 0.02 cm^3 whole blood in 5cm^3 of distilled or deionized water. The absorbance of hemoglobin and methemoglobin were measured at 540 nm and 630 nm respectively [16].

Determination of Ldh Activity of Sickle Cell Blood: The determination of LDH activity was carried out by the methods of DGKC technique. The methods of [18, 19] were used for the analysis. The reagent consists of a kit, 20 X 3 ml (Ref 99, 35 00) for 20 mono tests or 120 tests, semi-micro contents.

(A). 20 vials of freeze dried NADH (B). 1 X 60 ml buffered substrate.

The method used was the semi-micro technique at 30°C. 500 µL of the working reagent was left for 2-3 minutes at a temperature of 30 °C. 20 µL of normal saline was added to 20 µL of the specimen (serum). After mixing, the stop watch was started, the mixture transferred to a measuring cuvette and the absorbance read after 1, 2, 3 and 4 minutes at a wavelength of 340 nm. This served as control. In the assay mixture, the normal saline was replaced with 20 µL of the standard antisickling agent. The change in extinction (ΔE/min) was determined for all readings and the mean value recorded.

U/L =ΔE/min X factor,

The factor at 30 0 C=4127

U/L= ΔE/min x 4127

The normal values for LDH at 30° C=160-320 U/L

RESULTS

Table 1 summarizes the ascorbic acid (Vit.C) concentration of the samples expressed in mg/100g of foodstuff. Table 2 shows the total free amino acid concentration of the ripe fruit juices expressed in g/100g. Table 3 displays the amino acid profile of the ripe fruit juices. Table 4 shows the rate of polymerization, the relative rate of polymerization and the relative percent inhibition in the presence of the fruit juices. Table 5 shows the Fe^{2+}/Fe^{3+} ratio of sickle cell blood in

Table 1: Total Vitamin C Concentrations and the pH Values of the Fruit Juices

Fruit Samples	pH at 25°C	Vol. (ml)	Vit.C mg/mL	Vit.C mg/kg	Vit.C mg/100g
A. *Citrus sinesis*	4.75± 0.1	240.0	2.46±0.0	590.40	59.04±0.1^a
B. *Carica papaya*	5.27±0.2	80.00	5.19±0.1	415.20	41.52±0.1^a
C.*Citrus paradisi*	4.61± 0.1	186.0	2.14± 0.1	398.04	39.80±0.0^a
D.*Citrus limon*	2.80± 0.0	160.0	1.01±0.1	161.92	16.20± 0.0^b
E.*Citrus aurentifolia*	2.20±0.0	180.0	1.52±0.1	273.63	27.36±0.1^b
F.*Citrus reticulata*	5.00±0.0	140.0	1.22± 0.1	170.80	17.08±0.0^b

The values in the table are the Mean ± S.D from triplicate (n=3) determinations. The values with the same superscript are significantly the same and significantly different from others at 95% confidence level or p <0.05.

Table 2: Total Free Amino Acid Concentration of the Fruit Juices

Fruit samples	Vol. of Extract(ml)	Amino acid conc.(mg/ml)	Total amino acid conc.(g/kg)	Total amino acid conc.(g/100g)
A. *Citrus sinesis*	240.0	10.27±0.2	2.47± 0.01	0.250±0.00
B. *Carica papaya*	80.0	16.22±0.1	1.30±0.0 0	0.130±0.00
C. *Citrus paradisi*	186.0	15.72± 0.1	2.92±0.01	0.290±0.01
D. *Citrus limon*	160.0	6.73±0.0	1.06± 0.00	0.110±0.00
E. *Citrus aurentifolia*	180.0	5.22±0.1	0.94±0.11	0.094±0.01
F. *Citrus reticulata*	140.0	6.21±0.2	0.87±0.10	0.087±0.01

The values in the table are Mean ± S.D from triplicate determinations.

Table 3: The Major Amino Acids Identified in the Ripe Juices by Thin Layer Chromatography (TLC)

Fruit samples	Amino acids identified
A. *Citrus sinesis*	Met, Asp, Ile, Lys, Asn, Arg
B. *Carica papaya*	Phe, Lys, Val, Leu
C. *Citrus paradisi*	Met, Ile, Phe,Lys, Asn, Arg
D. *Citrus limon*	Asp, Arg, Val, Leu
E. *Citrus aurentifolia*	Asp, Ala Val,, Phe
F *Citrus reticulata*	Leu, Ala, Val, Phe, Asp

Table 4: The Rates of Polymerization and Relative Percent Polymerization of Sickle Cell Hemoglobin (HbSS) at 100µM Ascorbic Acid Equivalence of the Ripe Fruit Juices

Fruit samples	Final Assay Conc.(µM)	Rate of polymerization	Relative percent polymerization	Relative percent inhibition /enhancement.
Control	----	0.0046±0.0	100.0	0.00
L-Phenylalnine	100	0.00019±0.0	4.13	95.87± 0.0
Ascorbic acid	100	0.0009±0.0	10.60	80.40±0.0
A. Citrus sinesis	100	0.0008±0.0	17.40	82.60±0.1
B. *Carica papaya*	100	0.0007±0.0	15.20	84.80±0.1
C_1. *Citrus paradisi*	100	0.0240± 0.0	522.0	-422.0±0.0
C_2 (Dialyzed)	100	0.00083±0.0	23.71	76.29±0.2
D_1. *Citrus limon*	100	0.0072±0.0	156.0	-56.0±0.0
D_2 (Dialyzed	100	0.00125± 0.0	35.71	64.29±0.2
E_1 *Citrus aurentifolia*	100	0.0500±0.0	1086.9	-986.9±0.0
E_2 (Dialyzed)	100	0.0025±0.0	71.43	28.57±0.0
F *Citrus reticulata*	100	0.00065± 0.0	14.13	85.87±0.0

The values in the table are the Mean ± S.D from triplicate determinations. Values in the table with the same superscript are statistically related and are different from others at p=0.05. The values in the relative inhibition/enhancement column with minus (-) signs, indicate enhancement of polymerization. The samples whose alphabets are subscripted are the sickling samples and those with subscript (1) were un-dialyzed while those with subscript (2) were the dialyzed equivalents.

Table 5: Effect of Ripe Fruit Juices on the Fe^{2+}/ Fe^{3+} Ratio of Sickle Cell Blood at a Concentration of 40µm Ascorbic Acid Equivalence of the Juices

Fruit samples	%Hb	%mHb	Fe^{2+}/Fe^{3+} ratio	% reduction/ increase
Control	93.00 ±0.0	7.00± 0.0	13.30 ±0.0	0.00±0.0
L-Phenylalanine	97.20 ±0.0[a]	2.80±0.0	34.71±0.0[a]	146.5±0.0[a]
Ascorbic acid	96.27±0.1[a]	3.73 ± 0.1	25.81±0.1[a]	83.31±0.1[a]
A. *Citrus sinesis*	95.00 ±0.0 [a]	5.00± 0.0	19.00 ± 0.0[a]	42.90±0.0[a]
B. *Carica papaya*	94.00 ±0.0[a]	6.00±0.0	15.67 ± 0.0[a]	17.80±0.0[a]
C. *Citrus paradisi*	80.40 ±0.0[b]	19.60 ±0.0	4.10 ± 0.0[b]	69.20 ±0.0[b]
D. *Citrus limon*	81.62 ±0.0[b]	18.38 ±0.1	4.44 ±0.1[b]	66.60±0.1[b]
E. *Citrus aurentifolia*	73.26 ± 0.1[b]	26.74 ± 0.1	2.74 ±0.1[b]	79.04±0.1[b]
F. *Citrus reticulata*	92.58 ±0.2[a]	7.42 ± 0.1	9.87±0.1[a]	25.79±0.0[a]

The values in the table are the Mean± S.D from triplicate (n=3) determinations. Values in the columns with the same superscript are significantly the same and different from others at 95% confidence level or p<0.05. Values with superscript 'a' in the last column indicaTE increase in the ratio while those with superscript 'b' indicate the contrary.

Table 6: Serum LDH Activity of Sickle Cell Blood in the Presence of Ripe Fruit Juices at a Concentration of 37 µm Ascorbic Acid Equivalence

	HbSS BLOOD(GROUP A) (n=20)			HbSS BLOO (GROUP I) (n=20)		
Fruit sample	ΔE/ min	U/L	% increase/ Reduction	ΔE/min.	U/L	% increase/ reduction
Control	0.104±0.01	429.21±0.0	0.00	0.100 ±0.00 4	12.70±0.0	0.00
L-Phenylalanine	0.045±0.02	185.72±0.0	56.73[a]	0.050±0.00	206.35±0.0	50.0 [a]
Ascorbic acid	0.053±0.01	218.73±0.2	49.04[a]	0.055±0.01	226.99±0.1	45.0[a]
A. *Citrus sinesis*	0.078±0.00	321.90±0.1	25.00[a]	0.076±0.00	313.65±0.1	24.0[a]
B. *Carica papaya*	0.062±0.02	257.53±0.1	40.00[a]	0.062± 0.00	255.87±0.2	38.0[a]
C. *Citrus paradisi*	0.125±0.02	523.63±0.2	22.00[b]	0.124 ±0.00	511.75±0.2	24.0[b]
D. *Citrus limon*	0.118±0.00	485.00± 0.0	13.00[b]	0.113±0.00	466.30±0.0	13.0[b]
E. *Citrus aurentifolia*	0.093±0.00	525.00±0.0	22.30[b]	0.135±0.01	530.00±0.0	28.4[b]
F. *Citrus reticulata*	0.065±0.02	272.00±0.0	36.63[b]	0.068±0.02	301.28±0.2	27.0[a]

The values in the table are the Mean ± S.D from triplicate (n=3) determinations. The values with the same superscript are significantly the same and different from others in the same column at 95% confidence level or $p<0.05$. In the last column, the values with the superscript (a) indicated reduction in LDH activity while those with superscript (b) indicate increase in the enzyme activity.

the presence of the juices. It also shows the percent improvement or reduction in the ratio. Table 6 depicts the LDH activity of two groups of SCD blood samples, A and I (n=20), 'n' being the number of blood samples from HbSS donors in each group. The relative percent increase or reduction in LDH activity was equally determined.

DISCUSSION

Ascorbic acid had previously been shown to possess antisickling property [20]. In this work, the fruit juices were found to be rich sources of vitamin C. Apart from this vitamin, phenylalanine an outstanding antisickling amino acid was identified in all samples by TLC. The fruit juices of orange (*Citrus sinesis*), mandarin (*Citrus reticulata*) and pawpaw (*Carica papaya*) exhibited profound antisickling effectiveness by inhibiting polymerization to the levels of 82.60%, 84.0% and 85.87% respectively. These values when compared with those of phenylalanine (95.87%) and Ascorbic acid (80.40%) were not significantly different. The un-dialyzed samples of grape (*Citrus paradisi*)-422.0%; lemon (*Citrus limoni*)-56.50% and sour lime (*Citrus aurentifolia*)-986.96% enhanced sickle cell gelation remarkably. These sickling juices equally increased lactate dehydrogenase (LDH) activity of sickle cell blood and reduced the Fe^{2+}/ Fe^{3+} ratio drastically. These effects are shown in tables 4, 5 and 6 respectively. It should be noted that these sickling fruit juices are equally rich in vitamin C; the action of the sickling fruit juices expresses some level of toxicity which might be deleterious to the sickle cell disease patient. Apart from being nutritive, some chemical compounds in fruit juices eg grape fruit which contain furancocoumarins-a compound that affect cytochrome P450 enzymes, altering the metabolism of drugs. A variety of drugs such as the statins used for treating high cholesterol levels, drugs for heart medications and antibiotics have their toxicities increased [21]. That these sickling juices are used in commercial fruit juices and concentrates and their bye-products are consumed globally as beverages and drinks constitute a pronounced environmental hazard to unsuspecting consumers some of them who may be sickle cell disease patients. Some workers have equally reported on a component of lemon' lemonene' and other similar compounds present in cloves and nutmeg as toxic to humans in large doses [22]. Although these sickling fruit juices lost this property on dialysis; it would appear that the sickling apparatus of these juices may be attributable to a small molecule diffusible through a semi-permeable membrane. Moreover, the lower pH values of these sickling juices favor the formation of deoxyhemoglobin S (deoxyHbS) via the Bohr effect, by decreasing the solubility of sickle cell hemoglobin, increasing the likelihood of the sickling of erythrocytes that contain HbS [23]. Most plants parts (roots, stems, leaves, flowers and others have been found to be good sources of antisickling agents and phytochemicals used in the management of sickle cell disease. For example, an extract of *Mangifera indica* bark has been found to be a good hematinic for the management of SCD [24]. Unripe *Carica papaya* seed extract has also been found to possess antisickling potential [25]. Some workers have found the extracts of bitter orange (*Citrus bergamot* or *Citrus aurentum*) to cause disturbances in animals especially cardiac and blood pressure effects due to the presence of octapamine, phenylephrine and their parent compound, Synephrine [26]. This action which expresses some level of toxicity

is similar to our observation on these sickling juices: (*Citrus paradisi*, *Citrus limon* and *Citrus aurentifolia* respectively in enhancing gelation, reducing the Fe^{2+}/Fe^{3+} ratio and increasing LDH activity in the serum of different groups of HbSS patients. The nutritional approach to the management of sickle cell disease is quite novel and promising. There has also been reports on the efficacy of some nutrients like amino acids, glucose, vitamins and minerals which are devoid of toxicity [27,28]. Many environmental agents have equally been implicated in the sickling phenomenon, such as sodium metabisulphite and sodium dithionite used by many industries as antimicrobial agent in the preservation of fruit juices and wines, yet, these potent deoxygenating agents contribute immensely to the health problems of sickle cell disease and other patients. Sickle cell disease patients should be advised to abstain from the consumption of beverages and fruit juices containing the sickling fruit samples and their by-products as well as other foods preserved with strong deoxygenating agents like sodium metabisulphite to avoid aggravating severe sickling episodes and complications.

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