HAEMATOLOGICAL RESPONSE TO INTAKE OF UNRIPE CARICA PAPAYA FRUIT EXTRACT AND THE ISOLATION AND CHARACTERIZATION OF CARICAPINO SIDE: A NEW ANTISICKLING AGENT FROM THE EXTRACT

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ABSTRACT

Phytochemical investigation of the ethyl acetate fraction of the methanol extract of the unripe fruit extract of Carica papaya led to the isolation and identification of a new antiscikling agent 8-[2-0-β-D-4, 5-anhydroglucotyl 1→2 glucopyranosyl carbonyl] dibenz [b, e] [1,4] dioxine-2-carboxylic acid (named as caricapinoside) which is reported for the first time from Carica papaya and from natural sources. The structure was elucidated by the application of MS, 1D-, and 2D-NMR spectroscopic analyses and by comparison with literature data.

Aqueous extract of unripe Carica papaya has been shown to possess antiscikling properties. The effect of intake of the extract on haematological parameters in sickle cell patients were investigated using standard techniques, by determining the values before and at intervals during intake of the extract.

The packed cell volume (PCV) values obtained at the end of the study were significantly higher (p<0.05) than the values before ingestion. The total white blood cell count (WBC) and differential leucocytes (neutrophils, lymphocytes, eosinophils, monocytes and basophils) and platelets counts were within the reference range before and during ingestion throughout the study. It was established that the aqueous extract of unripe Carica papaya has no adverse but beneficial effect on haematological parameters in sickle cell patients.

Keywords: phytochemical, haematological, antiscikling agent, unripe carica papaya, caricapinoside

INTRODUCTION

Sickle cell disease is an inherited disorder that occurs as a result of substitution of the amino acid, glutamic acid with valine at the sixth position on the beta chain of haemoglobin. Traditional concepts of sickle cell pathophysiology ascribed all features of disease to sequential effects of the adenine-guanine nucleotide substitution in the sixth codon of the globin gene, substitution of valine for glutamic acid on the outer surface of the HbS molecule, reduced solubility and polymerization of HbS when deoxygenated, sickling and poor deformability of polymer-containing erythrocytes, and occlusion by sickle red cells of the microvasculature.

Carica papaya (family Caricaceae) originated in Central America. It is an interesting tree in that the male and female parts exist in different trees. The fruits, leaves, seeds and latex are used medically. Its main medicinal use is a digestive agent; it is prescribed for people who have difficulty digesting protein and is used to break up blood clots after surgery, and this is due to the presence of enzyme papain in the plants latex. The latex from the trunk of the tree is also used as a local anesthetic to induce menstruation, flowers have also been used for jaundice and inner bark used for sore throat.

Antisickling activity of Carica papaya aqueous extract has been established. The antiscikling agent was found to reside in the ethylacetate fraction of the extract. Toxicity studies on aqueous extract of unripe Carica papaya in Wistar albino rats revealed normal liver and kidney function tests, it was also shown that it has no toxic effect on haematological parameters. Normal liver and kidney functions were obtained in sickle cell patients of different age groups who ingested the aqueous extract for a period of six months.

The antiscikling and non-toxic effect of aqueous extract of unripe Carica papaya is well established except on haematological parameters in humans, isolation and characterization of the active ingredients has also not been determined, hence the present study is designed to determine the effect of ingestion of Carica papaya on haematological parameters in humans, isolate and characterize the active ingredient in unripe Carica papaya fruit.

MATERIALS AND METHODS

Authentication and preparation of unripe Carica papaya fruit extract

Mature fresh unripe Carica papaya fruit was obtained in a local garden in Ile-Ife, and was authenticated at the herbarium of the Botany Department, Obafemi Awolowo University, Ile-Ife, the herbarium number is 14729. Mature fruit was identified by the colour of the seeds inside the fruit; when matured and unripe, the seeds were brown, immature fruit had whitish seeds while ripe fruit had black seeds.

Preparation of fruit extract for oral ingestion

Mature fresh unripe Carica papaya fruit was plucked, peeled and the cream coloured seeds inside discarded, 100g of the fruit was immersed in 100ml of water and incubated at room temperature for 72 hours. The extract was sieved into a clean bottle and kept in the refrigerator until use.

The parents of the sickle cell children/adult patients gave their informed consent. The ethical approval for the study was obtained from ethics and research committee of Obafemi Awolowo University Teaching Hospital Complex, Ile-Ife.

Sources and collection of blood samples

Sources

Forty five sickle cell disease patients, whose hemoglobin type has been confirmed to be SS, were recruited for the study.

Category of patients

The patients were grouped into three on the basis of their age. There were 15 patients in each group.

Category one

Children (2 to< 6 years, x 5.2): These patients took one teaspoonful (5ml) of extract of unripe pawpaw fruit three times a day for 6 months.
Category two
Children (6 to < 12 years, x 9.7): These patients took two teaspoonsful (10ml) of extract of unripe pawpaw fruit three times a day for 6 months.

Category three
12 years and above, (x 21.4): These patients took three teaspoonsful (15ml) of extract of unripe pawpaw fruit three times a day for 6 months.

Collection of blood samples
Ten milliliters (10ml) of blood was collected from each of the patients through clean venepuncture before extract ingestion. 5.0ml was dispensed into EDTA bottle for haematological analysis, 2.25ml into 0.25ml of sodium citrate (3.8%) for coagulation studies and the remaining 2.75ml into ETA plate for haematological analysis.

Blood collection was repeated 24 hours, 1 week, 2 weeks, 3 weeks, 1 month, 2months, 3months, 4months, 5 months and 6 months after daily ingestion of the extract throughout the period of the study.

Blood samples were analyzed on the day of blood collection.

Haematological analysis
Packed cell volume (PCV), white blood cell (WBC) count and platelets were determined by ADVIA 60 Haematology system (Auto analyzer). Differential leucocyte count and blood cell morphology were performed manually after Leishman staining technique 19.

Statistical analysis
The mean and standard deviation and the level of significance for the differences between means were computed by students test SPSS 6.

General Experimental Procedures
Solvents used for extraction and chromatography included hexane, dichloromethane, ethyl acetate and methanol and were redistilled. Gel filtration was achieved using Sephadex LH-20. Adsorption column chromatography (cc) was performed with silica gel (Kieselgel 60 ASTM 230-400 mesh, 0.040-0.063mm). Thin Layer Chromatography (TLC) analysis was done using analytical silica gel 60 GF254+366 pre-coated alumina plates (Merck, 0.25 mm thick).

The results showing on TLC plates were visualized under UV light (254 nm) and by use of H2SO4/vanillin and sulfuric acid (H2SO4) spray reagents. The 1H-NMR and 13C-NMR spectra were recorded at 600 MHz at 150 MHz, on Bruker Avance DRX 600 spectrometers and ESI-MS were done on Finnigan LTQ Deca at the University of Botsswana, Botswana. Chemical shifts are given in ppm in δ values relative to TMS.

Extraction and solvent partitioning
The unripe Carica papaya fruits were peeled and the seeds inside discarded. The fruits (3kg) were cut into pieces and extracted with 5 litres of methanol at room temperature for 72 hours and the extract concentrated to dryness in vacuo on a rotary evaporator. The crude extract 200g was suspended in distilled water and partitioned with ethyl acetate and n-butanol successively, which in turn concentrated to dryness in vacuo. The ethyl acetate and n-butanol fractions were coded PEAF and PB respectively.

Fractionation of PEAF on Sephadex LH-20
The ethyl acetate (EtOAc) fraction, PEAF (6.7 g) was dissolved in a minimum amount of CHCl3-MeOH (80:20) and fractionated on a Sephadex LH-20 column previously equilibrated with the same solvent mixture. Elution was effected using a gradient of CHCl3-MeOH (80:20; CHCl3-MeOH (70:30); CHCl3-MeOH (50:50) resulting in four different fractions coded P1a, P1b, P1c and P1d.

Fractionation of P1d silica
P1d (108 mg) was fractionated on silica gel using a gradient of n-hexane (100%), n-hexane-ethyl acetate (50:50); ethyl acetate (100%) resulting in four fractions coded P2a, P2b, P2c and P2d (1).

RESULTS
Tables 1, 2 and 3 show the effect of ingestion of aqueous extract of unripe Carica papaya fruit on haematological profiles in sickle cell patients. In all age groups, there was significant increase (p<0.05) in PCV. The total WBC count and differential cell count fluctuated between significant increase or decrease (p<0.05) and no significant difference (p>0.05) in all age groups throughout the study; the same was observed for platelet counts. Table 4 and Figure 5 show NMR spectroscopic data and the structure of the antisickling agent respectively.

Table 1: Haematological response to intake of extract of unripe carica papaya in Sickle cell patients (2 to < 6 years x 5.2)
Table 2: Haematological response to intake of extract of unripe *carica papaya* in Sickle cell patients (6 to <12 years x 9.7)

<table>
<thead>
<tr>
<th></th>
<th>Zero hour</th>
<th>24 hours later</th>
<th>1 week after</th>
<th>2 weeks after</th>
<th>3 weeks after</th>
<th>1 month after</th>
<th>2 months after</th>
<th>3 months after</th>
<th>4 months after</th>
<th>5 months after</th>
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<tr>
<td>PCV</td>
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<td>23.27</td>
<td>24.53</td>
<td>25.00</td>
<td>25.33</td>
<td>26.00</td>
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<td>26.33</td>
<td>26.87</td>
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<td>27.07</td>
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<tr>
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<td>7288.67</td>
<td>6996.67</td>
<td>6580.00</td>
<td>7356.67</td>
<td>7533.33</td>
<td>7273.33</td>
<td>7520.00</td>
<td>6876.67</td>
<td>7450.00</td>
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<td>46.80</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>2.53</td>
<td>1.50</td>
<td>2.00</td>
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<td>2.23</td>
<td>2.08</td>
<td>1.55</td>
<td>1.67</td>
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<td>Lymphocytes</td>
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<td>49.00</td>
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<td>52.00</td>
<td>50.67</td>
<td>51.73</td>
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<td>51.53</td>
<td>51.60</td>
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<td>Monocytes</td>
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<td>0.91</td>
<td>0.85</td>
<td>1.06</td>
<td>1.30</td>
<td>0.79</td>
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<td>Platelets</td>
<td>195866.0</td>
<td>202733.0</td>
<td>189733.0</td>
<td>192400.0</td>
<td>187600.0</td>
<td>180266.0</td>
<td>173200.0</td>
<td>176400.0</td>
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<td>179466.0</td>
<td>198566.0</td>
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n = 15

Table 3: Haematological response to intake of extract of unripe *carica papaya* in Sickle cell patients (12 years and above x 21.4)

<table>
<thead>
<tr>
<th></th>
<th>Zero hour</th>
<th>24 hours later</th>
<th>1 week after</th>
<th>2 weeks after</th>
<th>3 weeks after</th>
<th>1 month after</th>
<th>2 months after</th>
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<th>4 months after</th>
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<td>26.27</td>
<td>26.53</td>
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<td>27.33</td>
<td>27.13</td>
<td>27.40</td>
<td>27.27</td>
<td>27.60</td>
<td>27.93</td>
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<td>WBC</td>
<td>5950.00</td>
<td>5543.33</td>
<td>5453.33</td>
<td>5666.67</td>
<td>5826.67</td>
<td>5716.67</td>
<td>5760.00</td>
<td>5890.00</td>
<td>5906.67</td>
<td>5866.67</td>
<td>5916.67</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>53.53</td>
<td>53.00</td>
<td>52.13</td>
<td>53.53</td>
<td>52.87</td>
<td>53.67</td>
<td>54.87</td>
<td>54.27</td>
<td>53.87</td>
<td>52.93</td>
<td>53.53</td>
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<tr>
<td>Basophils</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>45.27</td>
<td>46.07</td>
<td>46.00</td>
<td>45.00</td>
<td>45.47</td>
<td>44.53</td>
<td>44.47</td>
<td>44.53</td>
<td>43.93</td>
<td>43.53</td>
<td>44.47</td>
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n = 15

Table 4: H- and 13C-NMR Spectral data for Compound 1 in CD3OD at 600 MHz

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<thead>
<tr>
<th>Ring</th>
<th>No.</th>
<th>1H</th>
<th>13C</th>
<th>HMBC</th>
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<tr>
<td>C-1</td>
<td>7.29</td>
<td>(d, 3.0)</td>
<td>151.34</td>
<td>121.12, 121.49, 148.64, 154.47, 173.93</td>
</tr>
<tr>
<td>C-1a</td>
<td>148.64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2</td>
<td>121.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2 (C=O)</td>
<td>173.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3</td>
<td>6.85 (d, 3.0, 9.0)</td>
<td>171.12</td>
<td>121.49, 115.34, 116.47, 154.47</td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>6.7 (d, 9.0)</td>
<td>116.47</td>
<td>121.12, 154.47, 148.64, 121.49</td>
<td></td>
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</table>

n = 15
The Physical and Spectroscopic data on the Isolated Compound.

### 4, 5-anhydroglucitol

| C-1' | 4.49 (d, 7.8) | 96.82 | 76.62, 67.62 |
| C-2' | 4.37 (dd, 7.2) | 76.62 | 96.82, 67.62, 39.62 |
| C-3' | 3.78-3.82 (m) | 67.62 | 67.62, 73.49 |
| C-4' | 3.19 (dd, 7.2, 15) | 39.62 | 73.49, 76.62 |
| C-5' | 3.29-3.32 (m) | 73.49 | 67.62, 96.82 |
| C-6' | 3.63-3.69 (m) | 61.38 | 73.49 |

| Glucose | C-1'' | 5.15 (bs) | 92.57 | 70.51, 72.47 |
| C-2'' | 3.63-3.69 (m) | 74.92 | 96.82, 71.59 |
| C-3'' | 3.78-3.82 (m) | 70.51 | 72.47, 71.59 |
| C-4'' | 3.78-3.82 (m) | 71.59 | 72.47, 70.51 |
| C-5'' | 3.29-3.32 (m) | 72.47 | 61.47, 72.47 |
| C-6'' | 3.85-3.87 (m) | 61.47 | 72.47, 71.59 |

**Coupling patterns and coupling constants (J) in Hz are given in parentheses.**

**Figure 1: Structure of the antisickling agent.**

**DISCUSSION**

The fact that the PCV rose slightly and gradually throughout the period of the study could be that there was no haemolysis of red blood cells which usually lead to low PCV values in the sufferers. The absence of haemolysis could be due to antisickling activities of the aqueous extract. The fundamental problem in sickle cell patients is the sickling of red blood which leads to haemolysis of red cells and hence reduction of PCV.

The values obtained for WBC, differential leucocytes count and platelets neither reduced nor increased during intake of the extract, an indication that the extract has no toxic effect on these blood cells, as the values were constantly within the reference range throughout the study period. No inclusions were observed in all the cells when blood film appearance was examined under the microscope in the stained blood film.

In conclusion, it was established from this study that ingestion of aqueous extract of unripe Carica papaya has no adverse but beneficial effect on cellular components of blood in sickle cell patients. However, this antisickling property of the crude extract of Carica papaya is not surprising since it has been reported that p-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid (Protocatechuic acid), 4-hydroxy-3-methoxybenzoic acid (Vanic acid) previously isolated from Mulbery (Morus alba), Lobelia sellofifolia, which forms the nucleus of the present new isolated compound (caricapinoside) are chemical constituents responsible for the antisickling activity of these plants and had also been used as a positive control in antisickling assays.

Many protocatechuic acid glucosides also have been reported found naturally. For example the protocatechuic 3-O-β-glucopyranoside is reported in Lobelia sellofifolia, protocatechic 4-O-β-glucopyranoside is reported isolated from Angiopteris bygodifolia and Blatta orientalis while protocatechic 4-
O-{4-[(4-methyl-β-D)-glucopyranosyl]-6-hydroxybenzoic acid (1) (from Lygodium japonicum27).

Elucidation of structure of the isolated constituent from Carica papaya

Compound 1 was obtained as a brown amorphous solid. The positive-ion ESI-MS exhibited a sodiated ion [M + Na]+ at m/z 603.38 which was consistent with the molecular formula C26H22O12.

The structure of this compound was determined by 1H and 13C NMR using H-H COSY, DEPT, HMBC, DEPT and HMBC for long range connectivities. The 1H NMR spectrum displayed 6 aromatic protons. The 1H-NMR spectra exhibited and indicated the presence of two ABX coupling systems (δ 6.70 - 7.29).

The presence of two sugar residues (two glucose in pyranose form) was deduced from the observation of two anomic carbon signals at 92.57 and 96.82. The attached protons of the glucopyranose were located at δ 5.15 and δ 4.49 d, J = 7.8 Hz.

The 13C NMR and DEPT NMR spectra showed 26 carbon signals composed of aromatic CH (6), oxygenated CH2 (2), non-oxygenated CH2 (1), oxygenated non-aromatic CH (9), and quaternary carbons (8). By the combination of 1H-NMR, 13C-NMR, DEPT, HMBC and HMBC spectral analysis, all carbon and proton signals were definitely assigned. In 13C NMR spectrum, the aromatic methenes at positions C-1 and C-9, C-3 and C-7, C-4 and C-6 appeared at δ 115.34, 155.47, 148.64, and 121.42 respectively while the one at positions C-1a and C-9a, C-2 and C-8, C-4a and C-6a, C-2 (C=O) and C-8 (C=O) appeared at δ 148.64, 121.49, 154.47, 173.93, 173.64 respectively.

Crucial 1H-13C long range correlations as observed and determined from HMBC spectrum included the proton at δ 7.29 (H-2') and H-2, C-3, C-8, C-2 (C=O), C-8 (C=O) and C-7 (δ 154.47, 121.12, 154.47), C-9 (δ 115.34, 121.49, 121.12, 154.47), proton at δ 6.70 (H-4 and H-6) to C-4a, C-6a, C-2, C-8, C-3, C-7, C-1a, C-9a (δ 148.64, 148.64, 121.49, 121.12, 148.64), proton at δ 6.65 (H-3 and H-7) to C-1 and C-9, C-2 and C-8, C-4 and C-6, C-4a and C-6a (δ 115.34, 121.49, 116.47, 154.47), C-6a (δ 121.12), C-7a (δ 173.93). Particularly diagnostic was the linkage between the dibenzo [b, e] [1, 4] dioxine-2-carboxylic acid and the sugar unit which was confirmed to be C-2' (C-8 (C=O) from the HMBC experiments that showed strong cross peaks between the proton at δ 6.43 (H-2') and C-8 (C=O) (δ 173.64) and H-2 (δ 39.62). Crucial was the glycosidic linkage between two glucopyranose units which was confirmed to be C-1' / C-2'' from the HMBC experiments that showed strong cross peaks between the proton at δ 3.63-3.69 (m) (m) (H-2') and C-1' (δ 96.82).

On the basis of these data (the 13C NMR, DEPT as well as HMBC and HMBC spectra) which have enabled the unequivocal assignments of the carbon signals, compound 1 was identified as 8 (2-0-β-D-4, S-anhydroglucitol 1→ 2 glucopyranosyl benzyol) dibenzo [b, e] [1, 4] dioxine-2-carboxylic acid (named as caricapinoside) which is reported for the first time from Carica papaya and from natural sources.

ACKNOWLEDGEMENTS

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REFERENCES


