International Journal of Phytomedicine 9 (2017) 38-42
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Comparative studies on antisickling properties of brown and green leaves of *Carica papaya* Linn. (Caricaceae)

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Abstract

Sickle Cell Disease (SCD) is characterised by non-covalent polymerisation of the haemoglobin under hypoxia conditions and this promotes red blood cell sickling. Inhibition of sickle cell haemoglobin polymerization is one of the areas of focus in the management of SCD. Dried *Carica papaya* leaves are used in traditional herbal remedies for the management of sickle cell anaemia; without specifying if it is the dried green or brown leaf.

This study was aimed at verifying the antisickling activity of the crude aqueous extract, crude methanol extract and fractions of dried brown and green leaves of *Carica papaya*.

The method used was the sickle cell haemoglobin polymerization inhibition experimentmeasured with the Ultra Violet (UV) spectrophotometer. Sodium metabisulphite was used as a deoxygenating agent while isotonic saline (0.9% NaCl) was used as the negative control and phenylalanine as a positive control. The results obtained showed that crude aqueous extracts of both the green and brown leaves exhibited high level of inhibition of sickled haemoglobin (HbSS) polymerization at200 mg/ml (97.76% and 93.25%), 100 mg/ml(95.89% and 97.93%) also, 50 mg/ml(97.89% and95.84%) respectively which compared favourably and significantly ($p<0.05$) with that of phenylalanine.

The summary of the antisickling activity of the crude extracts and fractions of both the green leaves and brown leaves of *C. papaya* is Crude aqueous>Crude aqueous methanol> Butanol>Chloroform>Ethyl acetate>Aqueous. This study showed that the extracts exhibited the potential of inhibiting polymerization of sickle cell haemoglobin thus would be beneficial in the management of sickle cell disease.

Keywords: *Carica papaya*, Sickle cell disease, crude aqueous extract, crude aqueous methanol extract and fractions.

Introduction

Sickle Cell Disease (SCD) is a multi-system disease associated with episodes of acute illness and progressive organ damage and it is one of the most common severe monogenic disorders worldwide [1]. It is a genetic disease caused by abnormal haemoglobin called sickle haemoglobin (HbSS), which polymerizes under deoxygenated condition and deforms the red blood cells into a 'sickle' shape [2]. SCD is characterized by premature breakdown of the red blood cells causing constant anaemia and occlusion of small blood vessels leading to excruciating body pains and other manifestations [3]. The treatment of SCD using orthodox approach has proved difficult and inefficient due to the genetic origin of the disease. Inhibition of sickle cell haemoglobin polymerization is one of the areas of focus in the management of SCA.

Carica papaya Linn. (Caricaceae) is an herbaceous succulent plant that possesses self-supporting stems [4]. The latex from the leaves has been used as antihelminths, antibacterial and for the production of papain which is used in food, textile and pharmaceutical industries [5]. An infusion of the mature leaves is also taken as an antidote for fever and malaria [6]. In Ivory Coast and Nigeria, water in which the young leaves have been crushed and squeezed is drunk three times daily for the treatment of hernia and urogenital infections [7]. Traditionally, the leaf extract was used as a tonic for the heart, analgesia and treatment of stomach ache [8]. *C. papaya* leaf methanolic extract has been reported to possess antisickling properties as they contain compounds that are capable of inhibiting and reversing the sickling of the red blood cells [9,10].

Materials and methods

Plant Material

DOI:10.5138/09750185.1961

The green leaves of *C. papaya* were collected from University of Lagos Botanical Garden while the dried brown of *C. papaya* leaves were collected from Department of Anatomy, University of Ibadan, Ibadan. The leaves were identified and authenticated at the University of Lagos herbarium where a voucher specimen - LUH 6033 was deposited for future reference. The leaves were oven dried separately, pulverized and stored in a container.

Preparation of plant extract

A decoction of 100 g of the dried powdered leaves was prepared. The resultant crude aqueous extract (CAE) was freeze dried using the LTE Freeze Drier (United Kingdom) till a powdered extract was obtained. The crude aqueous methanolic extract (CAME) was prepared using 350 g of the leaves by defatting with petroleum ether 60-80^{0}C, extracted exhaustively with aqueous methanol (1:3 v/v) using the soxhlet apparatus (Thermo Scientific UK) and then concentrated in a rotary evaporator (HeidolphLaborota 4010 digital, Germany). It was then fractionated successively with n-hexane, chloroform (CF), ethyl acetate (EAF), butanol (BF) and water (AF) in order of increasing polarity.

Collection of blood sample and Preparation of erythrocyte haemolysate Confirmed sickle cell (HbSS) blood was collected into an EDTA (Ethylene Diamino Tetra-acetic Acid) bottle from Haematology Clinic of the Lagos University Teaching Hospital, Idi-araba, Lagos, Nigeria.

Blood samples were centrifuged at 2000 rpm for 10 minutes using the HeraeusLabofuge 200 Centrifuge (Thermo Scientific, Germany). Following careful siphoning of the plasma with Pasteur pipette, the erythrocytes were washed three times with a volume of isotonic saline (0.9%) equivalent to the siphoned plasma. The samples were then centrifuged each time at a speed of 2000 rpm to remove the supernatant. The resulting erythrocytes were then suspended in a volume of isotonic saline equivalent to the siphoned plasma. The erythrocyte suspension was then frozen at 0ºC, and subsequently thawed before the experiment to produce haemolysates.

Sickle cell haemoglobin polymerization inhibition experiment

The underlying principle is that HbSS undergoes polymerization when deprived of oxygen, transiting to deoxyHbSS molecules; sodium metabisulphite was used as a deoxygenating agent [11]. 4.8 ml of freshly prepared 2% sodium metabisulphite ($Na_2S_2O_5$), 0.1 ml of HbSShaemolysate and 0.1 ml of isotonic saline (0.9% NaCl) the negative control (NC) was added into a test tube. The mixture was mixed thoroughly on a votex mixer, transferred into a cuvette and optical density measured using the spectrophotometer. Absorbance (O.D) values were taken at one (1) minute interval for 10 mins at 700 nm to determine the rate of polymerization; this served as the negative control. For the test assay, 0.1 ml of the test compound (*C. papaya* of different concentrations, 200 mg/ml, 100 mg/ml, 50 mg/ml of the extracts and fractions) was used in place of 0.1 ml isotonic saline and the procedure repeated as above. 10 mM of the antisickling amino acid, phenylalanine (Phe) was used as a positive control (PC) in place of the test compound [12] and the test conducted accordingly.

Statistical Analysis

The rates of haemoglobin polymerization for the extracts and fractions were estimated by calculating the tangent of a plot of average change in extinction or change in optical density (OD_{700} nm) versus time in minutes using the Graph pad prism software 5.0. The rates were equally expressed as percentages with respect to control. This gives the relative percent (%) inhibition. Results were considered to be statistically significant at $p<0.05$

Results

Results of the analyses are presented in tables 1- the percentage yield of all the fractions, table 2 - the rates of polymerization, the relative percent polymerization and the relative percent inhibition of HbSS of the crude extract and fractions of fresh green leaves of *C. papaya* and table 3 - the rates of polymerization, relative percent polymerization and the relative percent inhibition of HbSS of the crude extract and fractions of dead brown leaves of *C. papaya*.

Table 1: Percentage yield of fractions of dried green and brown leaves of *C. papaya*

Fraction	Yield for fresh green leaves (%)	Yield for dead brown leaves (%)
n hexane	0.15	0.51
Chloroform	0.40	1.40
Ethyl acetate	0.08	0.30
Butanol	0.90	3.20
Aqueous	11.78	41.23

Table 2: The rates of polymerization and the relative percent inhibition of HbSS of the green leaves of *C. papaya*

Extracts /Fractions (mg/ml) Control	Rate of Polymerization	Relative percent polymerization	Relative percent inhibition
NC- NaCl 0.9%	0.1160	100.00	0.00
PC -Phe 10mM	0.0131	11.32	88.68
CAE - 200	0.0026	2.24^{c}	97.76^{b}
100	0.0078	6.73^{c}	93.27^{b}
50	0.0048	4.11^{c}	95.89^{b}
CAME - 200	0.0106	9.12^{c}	90.88^{b}
100	0.0074	6.35^{c}	93.65^{b}
50	0.0140	12.08^{b}	87.92^{a}
CF - 200	0.0137	11.81^{b}	88.19^{a}
100	0.0260	22.39^{b}	77.61^{c}
50	0.0468	40.30^{b}	59.70^{c}
EF - 200	0.0207	17.83^{b}	82.17^{a}
100	0.0763	65.75^{b}	34.25^{c}
50	0.0886	76.34^{b}	23.66^{c}
BF - 200	0.0216	20.94^{b}	79.06^{c}
100	0.0154	13.28^{b}	86.72^{a}
50	0.0080	6.92^{c}	93.08^{b}
AF - 200	0.0429	36.95^{b}	63.04^{c}
100	0.0795	68.50^{b}	31.49^{c}
50	0.0783	67.52^{b}	32.48^{c}

Table 3: The rates of polymerization and the relative percent inhibition of HbSS of the dried brown leaves of *C. papaya*

Extracts /Fractions(mg/ml) Control	Rate of Polymerization	Relative percent polymerization	Relative percent inhibition
NC- NaCl 0.9%	0.1160	100.00	0.00
PC - Phe 10mM	0.0131	11.32	88.68
CAE - 200	0.0024	2.07^{c}	97.93^{b}
100	0.0025	2.11^{c}	97.89^{b}
50	0.0048	4.16^{c}	95.84^{b}
CAME - 200	0.0185	15.97^{b}	84.02^{a}
100	0.0102	8.75^{c}	91.25^{b}
50	0.0134	11.57^{a}	88.43^{a}
CF - 200	0.0355	30.62^{b}	69.38^{c}
100	0.0363	31.25^{b}	68.75^{c}
50	0.0331	28.53^{b}	71.47^{c}
EF - 200	0.0186	16.02^{b}	83.98^{a}
100	0.0489	42.15^{b}	57.84^{c}
50	0.0956	82.39^{b}	17.60^{c}
BF - 200	0.0189	16.25^{b}	83.75^{a}
100	0.0208	17.88^{b}	82.11^{a}
50	0.0155	13.34^{b}	86.66^{a}
AF - 200	0.0628	54.13^{b}	45.87^{c}
100	0.0786	67.72^{b}	32.28^{c}
50	0.0770	66.37^{b}	33.63^{c}

Non-significantly ($p>0.05$)^{a}, Significantly higher ($p<0.05$)^{b}, Significantly lower ($p<0.05$)^{c} than the positive control

Discussion

Inhibition of sickle cell haemoglobin polymerization is one of the areas of focus in the management of SCD thus, it has been hypothesized that antisickling drug or agent should significantly inhibit polymerization of the abnormal sickle haemoglobin HbS [13]. For the green leaves, the CAE exhibited high level of inhibition of HbS polymerization of 97.76%, 93.25% and 95.89% at all concentrations - 200mg/ml, 100mg/ml, 50mg/ml respectively which compared favorably and significantly ($p<0.05$) with that of Phe (Table 2), a well-researched standard antisickling agent [14,15]. The CAME also exhibited a high level of inhibition of HbS polymerization of 90.88% and 93.65% at 200mg/ml and 100mg/ml respectively while only the lowest concentration – 50mg/ml of the BF exhibited high level of inhibition- 93.08% which compared favorably and significantly ($p<0.05$) with that of Phe (Table 2). The CAME (50mg/ml), CF (200mg/ml), EF (100mg/ml) and BF (100mg/ml) exhibited high level of inhibition of HbS polymerization of 87.92%, 88.19%, 82.17% and 86.72% respectively though it was non-significant ($p>0.05$) when compared with that of Phe (Table 2).
For the brown leaves, the CAE exhibited high level of inhibition of HbS polymerization of 97.93%, 97.89% and 95.84% at all concentrations – 200mg/ml, 100mg/ml, 50mg/ml respectively while the CAME exhibited high level of inhibition 91.25% at 100mg/ml which compared favorably and significantly ($p<0.05$) with that of Phe (Table 3).
The CAME (200mg/ml, 50mg/ml), EF (200mg/ml) and BF (200mg/ml, 100mg/ml, 50mg/ml) exhibited high level of inhibition of HbS polymerization of 84.02% , 88.43%, 83.98%, 83.75%, 82.11% and 86.66% respectively though it was non-significant ($p>0.05$) when compared with that of Phe (Table 2).
These results, coupled with the over 50% inhibition potential (although significantly, $p<0.05$, lower than that of Phe) observed in the CF of both the fresh green and the brown leaves may be due to the polar nature of these fractions and their ability to diffuse into the haemoglobin molecule to bind at the heme pocket, thereby obstructing the 'sticky patches' of the sickle cell Hb molecules [11]. This will prevent polymerization of Hb molecules into long fibers that would have caused deformation into sickle shapes of the normal disc biconcave shape of RBCs [16]. On the other hand, the AF of both the green and brown leaves at 50mg/ml exhibited a significantly ($p<0.05$) low percent inhibition of polymerization in comparison with that of the positive control. This may be attributed to the fact that the antisickling activity is concentration-dependent. The 200mg/ml concentration of *C. papaya* leaf fractions were more effective in inhibiting sickling than other concentrations. *C. papaya* leaf crude extracts exhibited the highest level of inhibition of HbS polymerization compared to the other extract fractions and positive control used. This finding is comparable to previous studies which have found plant crude extracts to be more effective than its various fractions [17]. The antisickling properties of the leaf could be concentrated in the polar and phenolic constituents of *C. papaya* as evidenced by the potent antisickling activities of the crude aqueous extract, crude aqueous methanol extract and butanol fraction. The summary of the antisickling activity of the crude extracts and fractions of the green leaves and dead leaves of *C. papaya* is CAE>CAME>BF>CF>EAF>AF. This study also supports the claims of previous studies on antisickling activity of phytomedicines which showed that aqueous methanol forms of extraction contained the active constituents responsible for their observed activity [11, 18, 19]. The use of sodium metabisulphite to induce sickling is probably a more drastic approach than what actually happens in the vascular system of humans [20]. It is therefore expected that the extracts may achieve more efficient sickle inhibition *in vivo*. The high level of inhibition of HbS polymerization exhibited by *C. papaya* leaf indicates that the extract may be used to attenuate SS cell sickling and establish their abilities to inhibit sickling under hypoxic conditions thus justifying their use in folklore medicine for SCD management.

Conclusion

The results obtained in this study showed that the extracts exhibited the potential of inhibiting polymerization of sickle cell haemoglobin thus they would be very beneficial in the management of sickle cell disease.

Authors' contribution

Conception and design of the study: GO Ajayi, OM Ogun
Performing of the experiments: GO Ajayi OM.Ogun
Data Analysis: OM Ogun
Contribution of reagents and materials: G O Ajayi, O M Ogun
Writing of manuscript: G O Ajayi, O M Ogun
Authors have approved the final manuscript.

Funding support

This study received no external financial support.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

Acknowledgements

Mrs. Afolabiof the Department of Pharmaceutics; Mr. Adelekeand Mr.Julius of the Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos are acknowledged for technical assistance.

Abbrevations

UV –Ultra violet, HbSS - sickle haemoglobin, CAE – Crude aqueous extract, CAME – Crude aqueous methanolic extract, CF – Chloroform fraction, EAF – Ethylacetate fraction, BF – Butanol fraction, AF – Aqueous fraction, PC – positive control, Phe– phenylalanine, NC – Negative control, NaCl – Sodium chloride, SCA – Sickle Cell Anaemia, SCD – Sickle Cell Disease, EDTA - Ethylene Diamino Tetra-acetic Acid, O.D – Optical Density, mM– millimole, rpm – revolutions per minute

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