British Biotechnology Journal
15(3): 1-7, 2016, Article no.BBJ.21696
ISSN: 2231–2927, NLM ID: 101616695

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Assessment of Antiproliferative Potential of *Hexalobus crispiflorus* (Annonaceae)

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SEO and AYT designed the study. Author AYT performed the statistical analysis. Author BOE wrote the protocol and wrote the first draft of the manuscript. Authors SEO and AYT managed the analyses of the study. Authors BOE and SEO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2016/21696
Editor(s):
(1) Ng Zhi Xiang, Department of Biomedical Sciences, Faculty of Medicine, MAHSA University, Malaysia.
Reviewers:
(1) Mario Bernardo-Filho, Universidade do Estado do Rio de Janeiro, Brazil.
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(5) Weiting Wang, Tianjin Institute of Pharmaceutical Research, Tianjin, China.
Complete Peer review History: http://www.sciencedomain.org/review-history/15920

Original Research Article

Received 29th August 2015
Accepted 30th November 2015
Published 25th August 2016

ABSTRACT

Background: *Hexalobus crispiflorus* root aqueous extract (HC) is used to treat breast cancer in Nigeria.
Aim: To evaluate the antiproliferative effects of HC using *Sorghum bicolor* seed radicle as test subject.
Materials and Methods: Ten millilitres each of one to seven mg/ml HC in distilled water was poured into 9 cm wide Petri dishes overlaid with cotton wool and filter paper. Twenty viable seeds

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of *Sorghum bicolor* (Guinea corn) were spread on each plate and incubated in the dark. The lengths (mm) of the radicles emerging from the seeds were measured at 48, 72, and 96 h. The control seeds were treated with 10 ml of distilled water containing no extracts. The experiments were carried out in triplicates. The mean radicle lengths (mm) and percentage inhibition were determined. HC was characterized using colour reactions and HPLC-UV-DAD.

Results: Phytochemical investigation revealed the presence of tannins, saponins, glycosides, alkaloids, flavonoids, terpenes and steroids. HPLC spectrum gave nine peaks with caffeic acid and rutin eluting at 5.22 and 7.76 minutes respectively. HC significantly ($P < 0.001$) inhibited *S. bicolor* seed growth over a period of 48 – 96 h against the control seeds. At 96 h, HC dose-dependently inhibited seed growth, gave inhibition of 35.41%, 40.67%, 58.24%, 61.34% and 63.68% for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml respectively with IC_{50} of 3 mg/ml. Methotrexate 0.176 mg/ml as positive control gave inhibition of 99.11% at 96 h.

Conclusion: This result indicates the propensity of HC to inhibit the proliferation of *S. bicolor* seed radicle, hence provide preliminary evidence for its use to treat breast cancer.

Keywords: Antiproliferative; Sorghum bicolor; cell growth inhibition; caffeic acid; rutin; Hexalobus crispiflorus.

1. INTRODUCTION

Cancer generally refers to a group of diseases that cause cells in the body to change and grow out of control. It can spread to other parts of the body through lymph and blood Breast cancer has been described as the most commonly diagnosed malignancy and the leading cause of cancer-related deaths of women worldwide [1]. Incidence of cancer is on the increase worldwide, with estimated 14.1 million new cancer cases in 2012; female breast, colorectal and stomach cancers accounted for over 40% of all cases diagnosed globally. Lung cancer accounted for 16.7% of all new cases in men [2,3].

Hexalobus crispiforus A. Rich belongs to the family annonaceae of plants. It is a tree of dense and fringing forest, attaining up to 30 m height by 1.7 m girth, with geographical distribution covering Guinea Bissau, Nigeria, Sudan and Angola. In Nigeria, it is known as “oji ogoda” (Igbo) and ‘apara joke’ (Yoruba). It has been reported to contain glycosides, saponins and steroids. It is used to treat skin diseases, eye diseases, fever and wound [4]. It is also known as “aga oji” or ‘uda egu’ in Uhunowerre / Igbo-eze South Local Government Area, South East Nigeria where a concoction of the root is used in treatment of breast cancer and other diseases.

Worldwide, breast cancer is by far the most common cancer in women accounting for 25% of newly diagnosed cancer. Cancer also accounted for more than 8.2 million deaths worldwide in 2012 [3]. Current treatment for cancer includes surgery, radiation therapy, chemotherapy, hormone therapy among others. In cancer chemotherapy, combination of drugs had been found to be more effective than one alone even though with their attendant toxicity problem. Hence the growing need for new anticancer drugs which are effective and less toxic. Interestingly, cancer patients who are burdened with drug-induced toxicity are getting help from complementary and alternative medicines that are plant-based.

Plants have been used for decades in treating numerous diseases including cancer. It has been estimated that 80% of people living in rural areas depend on medicinal plants for their primary health care needs [5]. Medicinal plants and natural products have played a significant role in the prevention and treatment of cancer through multiple therapeutic effects which include inhibition of cancer activating enzymes and hormones, stimulation of DNA repair mechanisms, enhancing production of protective enzymes, antioxidant and immune boosting activities [6,7].

The aim of this study is to evaluate the phytochemical and antiproliferative effects of the root aqueous extract of *H. crispiflorus* (HC) used in Nigeria for the treatment of breast cancer, on rapidly growing cells such as *Sorghum bicolor* seed radicles.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Unless otherwise stated all chemicals and reagents were of analytical grade and purchased from Sigma Aldrich (Germany). Acetonitrile is

HPLC grade purchased from Sigma Aldrich (Germany).

2.2 Plant Materials

Hexalobus crispiflorus was harvested in the month of August, 2014 from Uhunowerre in Igbo-eze South Local Government Area of Enugu State, Nigeria. It was identified and authenticated by Mr Felix Nwafor at the Herbarium of the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State where voucher specimen (Inter CEDD/921) was deposited.

2.3 Plant Preparation and Extraction

The fresh roots of *H. crispiflorus* were chopped into small pieces and air-dried at ambient temperature (28-30℃) to constant weight. The dried plant sample was pulverized. Then 6.6 g of powdered sample was weighed and extracted by hot water (500 ml) infusion in an air tight container for 24 h. The resultant mixture was vacuum filtered using Whatman No. 1 filter paper. The filtrate was dried at 80℃ on a water bath to yield *H. crispiflorus* root aqueous extract (HC).

2.4 Phytochemical Analysis

Phytochemical analyses were conducted on the *H. crispiflorus* root extract (HC) for secondary metabolites such as tannins, alkaloids, anthraquinones derivatives, saponins, glycosides, cardiac glycosides, terpenes, steroids and flavonoids using method described by Harborne [8].

2.5 High Performance Liquid Chromato-graphy Analysis

The chromatographic system includes Shimadzu HPLC system consisting of Ultra- Fast LC-20AB prominence equipped with SIL- 20AC auto-sampler; DGU-20A3 degasser; SPD-M20A UV-diode array detector (UV-DAD); column oven CTO-20AC, system controller CBM- 20Alite and Windows LC solution software (Shimadzu Corporation, Kyoto Japan); column, VP-ODS 5µm and dimensions (150 x 4.6 mm). The chromatographic conditions included mobile phase solvent A: 0.2% v/v formic acid and solvent B: acetonitrile; mode: isocratic; flow rate 0.6 ml/min; injection volume 5 µl of 50 mg/ml solution of HC in the mobile phase; detection was at UV 254 nm wavelength. Reference standards, Rutin, Quercetin, Caffeic acid, Ferulic acid and Apigenin (Fluka, Germany) 50 µg/ml in methanol were analysed separately under the same condition as the extract (HC) [9]. The HPLC operating conditions were programmed to give the following: solvent B: 20% and column oven temperature 40℃. The total run time was 20 minutes.

2.6 Experimental Plant

The experimental plant, *Sorghum bicolor* (Guinea corn) seeds was purchased from Dumez small market along Abuja-Kaduna express way, Niger State and identified and authenticated by Mr. Muazzam Ibrahim of NIPRD Abuja. The seeds viability tests were determined by placing them inside a beaker containing water. The seeds that floated were discarded while the totally submerged seeds were cleansed with methylated spirit and dried for usage.

2.7 Determination of Growth Inhibitory Effects

The modified methods of Ayinde et al. [10] and Chinedu et al. [11] were used for this study. HC (200 mg) was dissolved in 10 ml of distilled water to obtained 20 mg/ml stock solution. Various concentrations (1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml) of HC were prepared. Methotrexate was made to a concentration of 0.167 mg/ml as positive control. Petri dishes were layered with cotton wool and filter paper (Whatman No. 1). Twenty (20) seeds of *S. bicolor* were placed in each of the Petri dishes. The control seeds were treated with 10 ml distilled water. The test seeds were treated with the different preparations of HC as the seeds in each specific Petri dish received 10 ml of a particular concentration (the seeds in one Petri dish were treated with 1 mg/ml concentration, seeds in another Petri dish received 2 mg/ml, another received 4 mg/ml and the next received 6 mg/ml, while the seeds in the last Petri dish received and 7 mg/ml). The seeds were incubated in a dark room and observed for growth after 24 h. The mean lengths (mm) of radicle emerging from the seeds were measured after 48, 72 and 92 h. The percentage inhibition was calculated as [(mean radicle length control – mean radicle length treated)/mean radicle length control] ×100. Percentage growth was calculated as 100 – % inhibition. Percentage inhibition and percentage growth at 48, 72 and 92 h for seeds

treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml of HC and the positive control methotrexate at 0.167 mg/ml are as shown in Table 1.

2.8 Statistical Analysis

The data obtained were expressed as mean ± standard error mean and analyzed using Graph pad prism (version 6). Two way analysis of variance was used to test for significance. $P<0.001$ was considered to be significant.

3. RESULTS

Extraction of 6.6 g of *H. crispiflorus* roots powdered sample by hot water infusion yielded 0.52 g (7.9% w/w) of the dried extract (HC). Phytochemical screening of the HC indicated the presence of tannins, alkaloids, saponins, cardiac glycosides, flavonoids, terpenes and steroids. Anthraquinones derivatives were not detected. A total of nine peaks were detected from the HPLC spectrum with retention times of 2.73, 3.14, 3.64, 4.16, 5.22, 5.74, 7.76, 9.02 and 12.83 min as shown in Fig. 1. Caffeic acid and rutin appeared at retention times of 5.22 and 7.76 minutes respectively.

In the HPLC spectrum of HC (Fig. 1), total of nine peaks were detected with retention times of 2.73, 3.14, 3.64, 4.16, 5.22, 5.74, 7.76, 9.02 and 12.83 min. Caffeic acid and rutin eluted at retention times of 5.22 and 7.76 minutes respectively. Detection was at UV 254 nm.

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3.1 Growth Inhibitory Effects of HC on *Sorghum bicolor* (Guinea corn) Seeds

There was an appreciable reduction on the length of radicles of *Sorghum bicolor* seeds treated with the various concentration of the extract. The seed radicle lengths increased with the incubation period of 24 – 96 h. A rapid and progressive growth was observed in the water control seeds radicle lengths. At 48 h, percentage seed growth inhibition was 26.82% for seeds treated with 1mg/ml of HC. Then at 96 h, the mean radicle lengths of the control seeds was 59.00 ± 0.42 mm while the mean radicle length of the seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml were 38.55 ± 3.65 mm, 35.45 ± 4.45 mm, 24.95 ± 3.21 mm, 23.10 ± 3.03 mm and 21.70 ± 3.38 mm as shown in Fig. 2, corresponding to percentage inhibitions of 35.41%, 40.67%, 58.24%, 61.34% and 63.68% respectively. Therefore, the inhibitory effect of HC was concentration-dependent.

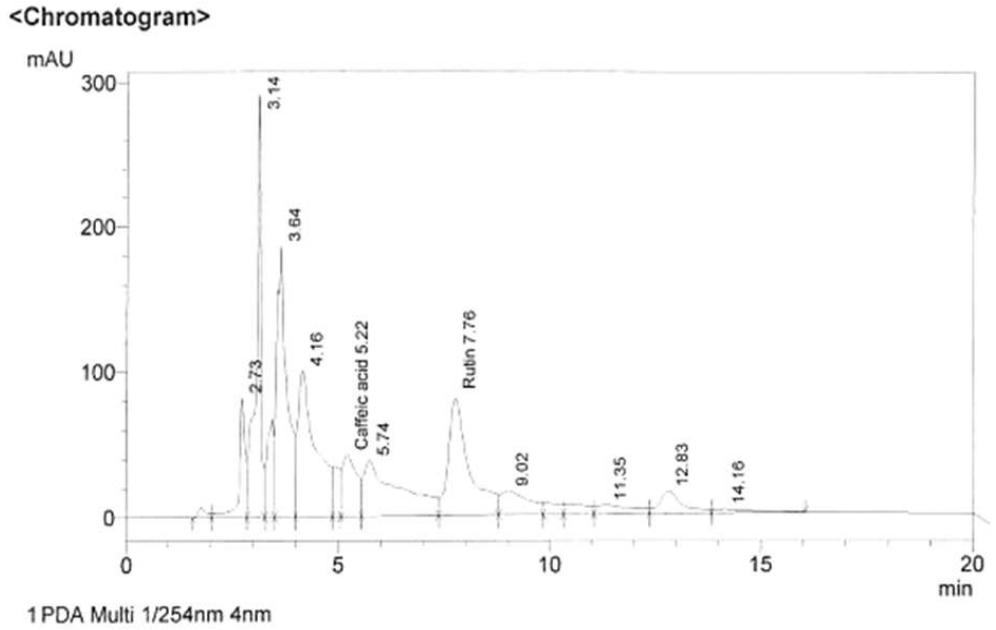

Fig. 1. HPLC spectrum of root aqueous extract of *Hexalobus crispiflorus* (HC)

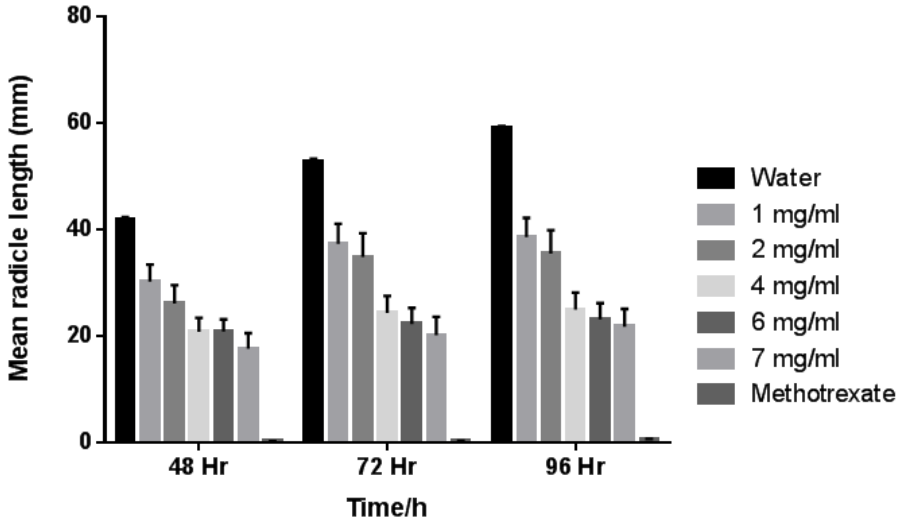

Fig. 2. Inhibitory effects of *Hexalobus crispiflorus* root aqueous extract (HC) on the growth of *Sorghum bicolor* seed radical

Table 1. Mean radical length, Percentage inhibition and percentage growth for *Sorghum bicolor* seeds treated with HC

Treatment	Mean radical length (mm)			Percentage inhibition*			Percentage growth†		
	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h
Control (H_2O)	41.90±0.41	52.80±0.46	59.00±0.42	0	0	0	100	100	100
Methotrexate	0.16±0.06**	0.18±0.07**	0.53±0.14**	99.61	99.68	99.11	0.39	0.32	0.89
HC (I mg/ml)	30.15±3.26***	37.30±3.73***	38.55±3.65***	26.82	33.31	35.41	73.18	66.69	64.52
HC (2 mg/ml)	26.10±3.45***	34.85±4.44***	35.45±4.45***	36.65	37.69	40.67	63.35	62.31	59.33
HC (4 mg/ml)	20.75±2.66***	24.35±3.17***	24.95±3.21***	49.64	56.46	58.24	50.36	43.54	41.76
HC (6 mg/ml)	20.85±2.26***	22.25±3.01***	23.10±3.03***	49.39	60.22	61.34	50.61	39.78	38.66
HC (7 mg/ml)	17.55±2.98***	20.10±3.51***	21.70±3.38***	57.40	63.70	63.68	42.60	36.30	36.32

: Percentage inhibition was calculated as [(mean radicle length control - mean radicle length treated)/mean radicle length control] ×100. †: Percentage growth was calculated as mean radicle length /time Percentage growth was calculated as 100 - % inhibition; methotrexate (0.167 mg/ml) used as positive control; n = 20. ** significantly ($P < 0.01$) inhibited S. bicolor seed growth at 48 h compared with the control (H_2O), *: HC significantly ($P < 0.001$) inhibited S. bicolor seed growth at 72 and 96 h compared with the control (H_2O).*

Inhibitory effects of HC on the growth of *Sorghum bicolor* seed radicle was determined for deferent concentrations: 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml. Radicle lengths (mm) were measured at 48, 72 and 96 h. Distilled water without HC was used as negative control, methotrexate 0.167 mg/ml was used as positive control, n = 20. methotrexate significantly ($P < 0.01$) inhibited *S. bicolor* seed growth at 48 h compared with the control (H_2O). HC significantly ($P < 0.001$) inhibited *S. bicolor* seed growth at 72 and 96 h compared with the control (H_2O).

Mean radicle length, percentage growth inhibition and percentage growth at 48, 72 and 92 h for seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml of HC, as well as the negative control, and methotrexate 0.167 mg/ml used as positive control are as shown in Table 1.

Inhibitory effects of HC on the growth of *Sorghum bicolor* seed radicle was determined for deferent concentrations: 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml. Radicle lengths (mm) were measured at 48, 72 and 96 h. Distilled water without HC was used as negative control, control n = 40, treated n = 20.

4. DISCUSSION

As a preliminary and preparatory assay to antiproliferative test on a cancer cell line system, the radicle lengths of fast growing seeds such as *Sorghum bicolor* has been utilized as a parameter for the testing of suspected anticancer

agents. Generally, cancer cells have a characteristic of fast proliferation, and this is also the case with meristematic cells of *S. bicolor* seeds when exposed to favourable conditions. [12] Hence, the use of the method for this study.

The phytochemical screening of HC showed the presence of tannins, alkaloids, saponins, glycosides, flavonoids, terpenes, steroids and anthraquinones were not detected. The HPLC spectrum of HC revealed the presence of caffeic acid and rutin at retention times of 5.22 and 7.76 minutes respectively (Fig. 1). Flavonoids have been reported to have relatively low toxicity compared to other metabolites like alkaloids. Flavonoids have also been referred to as 'natural biological response modifiers' because of the strong experimental evidence of their ability to modify the body's reactions to allergens, viruses and carcinogens. Flavonoids have anti-allergic, anti-inflammatory, antimicrobial and anticancer activities [13]. Saponins have been reported to have antioxidant, anticancer and anti-inflammatory activities and tannins to have antibacterial, antiviral and anti-tumor activities [14]. Generally, the pharmacological properties of medicinal plants depend on their secondary metabolites constitution [15,16]. Essential oil of *Hexalobus crispiflorus* from Cameroonian had been reported to possess anti-plasmodial activity against W2 strain of Plasmodium falciparum [17].

In this study, the results of the growth inhibitory effects of HC on *S. bicolor* (guinea corn) seeds showed a significant dose-dependent inhibition when compared with the control at 48, 72 and 96 h. The length of radicles of the seeds treated with different concentrations of the extract showed consistent and remarkable reduction when compare with the control throughout the experiment (Fig. 2). This is indicative of the antimitotic and antiproliferative effects of the phyto-constituents of HC. At 96 h, the *S. bicolor* seeds treated with 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml and 7 mg/ml showed 35.41%, 40.67%, 58.24%, 61.34% and 63.68% growth inhibitions respectively and the positive control methotrexate gave 99.11% growth inhibition (Table 1); indicating the growth inhibitory effect of HC on *S. bicolor* seed radicles to be concentration-dependent.

5. CONCLUSION

The aqueous extract of *Hexalobus crispiflorus* root (HC) exhibited growth inhibitory effects on fast proliferating cells (*S. bicolor* seed radicle). Hence, by extension it can inhibit cancerous cells. This study provided preliminary evidence that supports the ethno medicinal use of *H. crispiflorus* root in the treatment of breast cancer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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