Journal of Scientific Research and Development (2023) Vol. 22 (1) 14-23

A bi-annual journal published by the Faculty of Science, University of Lagos, Nigeria
http://jsrd.unilag.edu.ng/index.php/jsrd

Phytochemical profile, proximate analysis and antioxidant activity of fruit extracts of *Citrullus colocynthis*

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(Received 11January 2023/Revised 12 March 2023/Accepted 12 April 2023)

Abstract
This study was aimed to investigate the phytochemical profiles of *Citrullus colocynthis* fruit extracts, assessing the nutritive value and examining the antioxidant properties. These properties were examined using standard protocols. The crude was extracted with 50% aqueous methanol, partitioned into fractions, purified with column chromatography and the eluted components were analyzed using Fourier Transform Infra-Red (FTIR). The majority of the phytochemicals discovered in *C. colocynthis* were tannins (183.4 mg/100g tannic acid), reducing sugar (107.3 mg/100g glucose), and phenolic compounds (47.95 mg/100g gallic acid). The fruit contains 81% moisture, 7% carbohydrate and 9% total ash from the proximate analysis. The hexane, ethyl acetate and butanol fractions of the crude extract when tested at a concentration of 100 μL/mL in a 1,1-diphenyl-2-picrylhydrazyl assay displayed high antioxidant effects comparable to that of ascorbic acid (84.24% inhibition). *C. colocynthis* offers a significant degree of promise for pharmaceutical companies to synthesise into potential drugs for the treatment of diseases spurred by reactive oxygen species.

Keywords: *Citrullus colocynthis*, Antioxidant Properties, Proximate Content, Phytochemicals

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Introduction

Medicinal plants are considered to be as important therapeutic agents for human as they contain multiple phytochemical and antioxidant compounds. Fruits are common natural sources of pharmaceuticals that comprise well-known bioactive chemicals with a wide spectrum of pharmacological actions. Fruit phytochemicals have been found to contain carbohydrates, alkaloids, fatty acids, glycosides, flavonoids and essential oils with antibacterial, anti-diabetic, anti-hyperlipidemic, anti-fertility, and antioxidant activities (Naqvi *et al.*, 2020, Guan *et al.*, 2021). These natural products are classed as primary and secondary metabolites based on their chemical structure, function, and distribution across the plant kingdom. In the domains of medicinal chemistry and pharmacognosy, a more restrictive definition that confines natural products to secondary metabolites is widely utilized (Bhat *et al.*, 2005). Secondary metabolites are usually coloured, scented, or flavourful molecules that mediate the interaction of plants as well as other species, providing general protective effects such as antioxidants or free radical scavenging, chemicals toxic to microorganisms and allelopathy (Seca and Pinto, 2019). During biological processes, reactive oxygen species (ROS) are produced naturally as a consequence of oxygen metabolism and play a vital role in cell signalling and homeostasis. These ROS can spike at periods of environmental stress such as ultraviolet or heat exposure (Lourenço *et al.*, 2019), and cell structures may be negatively effected as a result. Cumulatively, this is referred to as oxidative stress. In essence, oxidative stress occurs when the balance of ROS generation surpasses the antioxidant capability of the target cell. Antioxidants are compounds that halt oxidative chain reactions by eliminating free radical intermediates (Xu *et al*., 2017). In recent years, there has been a noticeable increase in the search for natural antioxidants, particularly those derived from plants. The chemical composition and bioactive potential of crop plants of the family Cucurbitaceae such as *C. colocynthis* (Bitter Gourd) to scavenge ROS is becoming a subject of intense research. Members of Cucurbitaceae possess with antioxidant activity, such as carotenoids, polyphenols, cucurbitacins, or citrulline that have a free-radical scavenging ability.

Some investigators have confirmed that *C. colocynthis* fruits are usually recognized for its wide range of medicinal uses as well as pharmaceutical and nutraceutical potentials. Khatibi and Jahanbakhsh 2011 provided strong evidence for the antibacterial and anticandidal activities of the *C. colocynthis* plant. Similarly, a combination of the seed methanol extract of the plant with 0.5 g/ml of the antifungal drug fluconazole has been demonstrated to be very effective against dermatophytes (Ouf *et al*., 2022).

Most often, the parts of plants used for medicinal purposes are fruits and/or seeds, though other parts of the plants can be used. For example, the leaves have a diuretic effect and are used to cure jaundice and bronchitis and the root may be used to treat breast inflammation, amenorrhea, rheumatism as well as ophthalmia and uterine pains (Singh *et al*., 2020). In many Asian and African countries, the fruit is traditionally used to cure microbial diseases, jaundice, inflammation, fever, diabetes, and gastrointestinal diseases (Hussain *et al*., 2014; Al-Ghaithi *et al*., 2004).

Despite its widespread medical use in Nigerian folk medicine, research into the composition and the health-promoting benefits of bioactive compounds found in the fruits is limited. The aim of this study was to identify the phytochemical contents and antioxidant activities of the fruit extract for potential application in the management of diseases that have been implicated to be caused by elevated ROS. The fruit extracts were also subjected to proximate analysis.

Materials and Methods

Plant material

The fruit was bought at a local market in Lagos, Nigeria. It was identified and authenticated at the herbarium of Botany Department, University of Lagos. The voucher number assigned was LUH 7994. It was cut into tiny pieces, air dried and pulverized.

Extraction from plant materials

The dried, pulverised fruit was subjected to the extraction process as described in a previous study[12] using the following approaches:

Methanol/Water Extraction (Maceration)

Dried fruit powder of *C. colocynthis* was soaked in two litres of 50 % methanol-water (1:1) for 72 hours with frequent agitations. The extract was filtered and traces of the solvent were evaporated with rotary evaporation at 40 ^{o}C. A bench top freeze dryer was used to dry the concentrated extracts. This process was carried out twice to increase the yield, in all 50.5 g of extract 1000 g of the pulverized fruit.

Partitioning of Crude Extract

Aqueous mixture of 50 % methanol/water extract of *C. colocynthis* was obtained by dissolving in water with the ratio of extract to water being 1:1. The mixture was then partitioned between the aqueous and the organic solvents (hexane, ethyl acetate, butanol). Non-polar hexane was used first for separation followed by slightly polar ethyl acetate and the very polar butanol was used last on the same solution. This was to separate the crude into three fractions which are non-polar, slightly polar and polar fractions.

Organic solvents -water separation of *Citrullus colocynthis*

The crude extract was dissolved in 30 ml of water for this preparation and the non-polar chemicals was removed as described previously (Osibote *et al*., 2021). The hexane and ethyl acetate fractions were air dried, while the butanol fraction was concentrated by rotary evaporation.

Thin Layer Chromatography

Thin layer chromatography was used to investigate the three fractions. Approximately 5 mL of each extract (100 mg/ml) was deposited on fluorescent silica gel plates (0.2 cm thickness, 60 F254), and migration was carried out using several solvent systems and processed as described previously (Osibote *et al*., 2021).

Column Chromatography

Elution of components was monitored with UV light at 254 and 366 nm in separation of the mixtures on silica gel packed column with solvent systems developed for TLC. The different components for each fraction were obtained. HCC (2 components), ECC (3 components), and BCC (2 components) were subjected to FTIR (Model: Perkin-Elmer spectrum 11) analysis was used to determine the functional groups present and to support the phytochemical screening results (Osibote *et al.*, 2021)

Phytochemical screening

The extract was also subjected to quantitative phytochemical analysis (Osibote *et al.*, 2021) (Harborne, 1998). Alkaloids (Dragendorff, Wagner, and Mayer Tests), reducing sugar (Fehling's Test), flavonoids (Shinoda's Test) (Ahmad *et al.*, 2018),tannins (Ferric Chloride Test), cardiac glycoside (Keller Killani's Test) (El-Olemy *et al.*, 1994)., phenolic compound (Lead acetate Test) (Singleton and Rossi, 1999), saponins (Emulsion Test), Steroids (Salkwoski's test) and terpenoids were detected using conventional procedures.

Proximate analysis

The proximate analysis was carried out on the fresh fruit to estimate the percentage composition, moisture content, total ash, crude protein, crude lipids, crude fibre and nitrogen free extracts present he extracts using method described by Olayinka and Etejere (2018).

Total Antioxidant Capacity

Total antioxidant capacity of the extract was determined by measuring absorbance at 695 nm after 90 minutes of incubation in sealed test tubes with 0.3 mL of a 1 mg/mL extract solution in methanol combined with 2.7 mL phosphomolybdenum reagent. Trolox (Sigma-Aldrich, St. Louis, MO, USA) equivalents (mg TE/g dry sample) were used to calculate the total antioxidant capacity (Wijewardhana *et al.*, 2019). Controls included were butylated hydroxytoluene (BHT; Sigma-Aldrich, St. Louis, MO, USA) and oligopin (DRT nutraceutical, Vielle-St-Girons, France).

Free Radical Scavenging Activity

The free radical scavenging activities were tested using DPPH (1,1- diphenyl-2-picrylhydrazyl) solution as described by Benariba *et al.* (2013) and nitric oxide radical scavenging assay.

Results

Phytochemical screening

The total percentage yield of the 50 % aqueous methanol extract of the fruit *C. colocynthis* was 5.5 %. The phytochemical profile of *C. colocynthis* is presented on Table 1. Tannins (183.4 mg/100mg) were the most abundant component, while steroids and cardiac glycosides were found in smaller quantities. The terpenoids not detected quantitatively may have evaporated since terpenoids are volatile.

Table 1: Phytochemical profile of *Citrullus colocynthis*

Test	Method	Qualitative	Quantitative	Unit
Alkaloids	Mayer's Test	-	15.34	(mg/100g)
	Dragenderoff's Test	-	-	
	Wagner's Test	+	-	
Saponins	Emulsion Test	++	31.11	(mg/100g diosgenin)
Reducing Sugar	Fehling's Test	+	107.3	(mg/100g glucose)
Cardiac glycosides	Keller Killani's Test	+	5.53	(mg/100g Digoxin)
Terpenoids	Liebermaan – Burchard's	+ +	-	-
Steroids	Salkowki's Test	+	4.61	(mg/100g cholesterol)
Tannins	Ferric Chloride Test	+++	183.4	(mg/100g Tannic acid)
Phenolic Compounds	Lead acetate Test	+++	47.95	(mg Gallic Acid Equivalent/100g extract)
Flavonoids	Shinoda's Test	+	116.93	(mg Quercetin Equivalent/100g extract)

Key: Heavily detected: + + +; moderately detected: + +; slightly +; not detected -

Proximate Analyses

The *C. colocynthis* fruit extract contains 81 % moisture, 7 % carbohydrate and 9 % total ash (Figure 1). The other components were only in minute quantities.

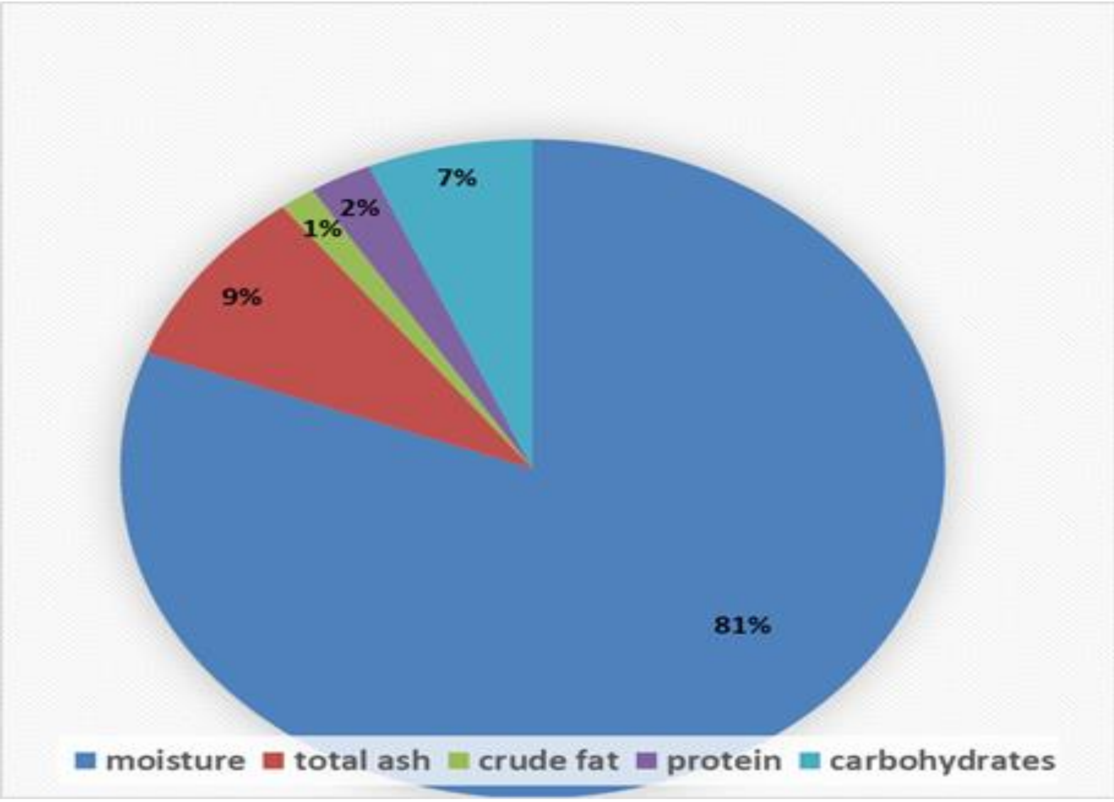

Figure 1: Proximate analysis of *Citrulluss colocynthis* fruit extract

Total antioxidant content of the extracts and Free radical scavenging activity

Table 2 reveals the total antioxidant content of the three fractions considered in this study. The ethyl-acetate fraction of the plant had the highest antioxidant and free radical scavenging activity of all the fractions, while the hexane and butanol fractions had the lowest antioxidant activity. The highest values for free radical scavenging activity were recorded in the ECC fraction, which was 16.72 mg/mg (ascorbic acid equivalent). Under the present experimental conditions, the HCC, ECC, and BCC lowered by 40 %, 62 %, and 59 % respectively, the DPPH signal, as compared to an 84 % decrease with 100 g/mL ascorbic acid. At a concentration of 100 µg/mL, the HCC, ECC, BCC, decreased under the present experimental condition, by 45.42 %, 53.91%, 47.70 %, the nitric oxide signal, as compared to 87.18 % decrease with 100 µg/mL ascorbic acid

Table 2: Total antioxidant activities of n-hexane, ethyl-acetate and butanol fractions of *Citrulluss colocynthis*

Sample	Nitric oxide Assay				DPPH Assay				TAC Ascorbic acid Equivalent (mg/mg extract)
	25 µg/mL	**50 µg/mL**	**75 µg/mL**	**100 µg/mL**	**25 µg/mL**	**50 µg/mL**	**75 µg/mL**	**100 µg/mL**	
HCC	14.9 ± 0.35	22.2 ± 1.09	38.7 ± 0.33	45.4 ± 1.68	20.3 ± 0.168	28.4 ± 1	34.5 ± 0.59	40.4 ± 0.57	13.0
ECC	22.0 ± 0.78	33.1 ± 0.02	42.8 ± 1.28	53.9 ± 0.58	40.2 ± 0.25	47.9 ± 0.47	56.4 ± 0.46	62.2 ± 0.6	16.7
BCC	18.7 ± 0.51	25.3 ± 0.92	33.9 ± 0.04	47.7 ± 0.4	30.5 ± 0.7	42.5 ± 0.47	55.8 ± 0.91	59.1 ± 0.51	11.8
Ascor-bic acid	73.1 ± 0.35	80.0 ± 0.41	84.5 ± 0.20	87.12 ± 0.26	64.3 ± 0.83	69.1 ± 0.32	74.5 ± 0.83	84.2 ± 0.77	

Abbreviation: **HCC -** , **ECC -** , **BCC -**

Column Chromatography and FTIR analysis

For the thin-layer chromatography, the solvent systems M:E:H 0.1:0.5:1 was established for HCC giving four (4) components with R_f. values 0.25, 0.30, 0.33, 0.38. E:D:M 1:1:0.1 was established for ECC three (3) components (R_f 0.25, 0.29, 0.33) and M:E:C 0.2:1:1 was established for BCC giving two (2) components (R_f 0.33, 0.38). The HCC fraction eluted two components with R_f values of 0.92 (0.0078 g) and 0.72 (0.0083 g), ECC eluted three components with R_f values of 0.86 (0.6 2 g), 0.65 (0.058 g), 0.51 (0.058 g). BCC eluted two components with R_f values of 0.71 (0.1971 g) and 0.89 (0.2004 g). Table 3 shows the functional groups identified by FTIR in the components eluted with column chromatography. Ethyl acetate fraction of *C. colocynthis* gave the highest number of components with bands corresponding to functional groups present in terpenoids and alkaloids which support bioactive activities in plants.

Table 3: The components eluted with column chromatography and the FTIR analysis

Fractions	Bands Present	Functional groups assigned
n -Hexane 1	3424.71cm^{-1}	OH stretching
	2918.35cm^{-1}	CH stretching
	2850.35cm^{-1}	CH_3 stretching
	17410 cm^{-1}	C=O stretching
	1463.83 cm^{-1}	CH_2 bending
	1373.78 cm^{-1}	CH_3 bending
	1242.80 cm^{-1}	C-O stretching
n-Hexane 2	3436.26 cm^{-1}	OH stretching
	2919.62 cm^{-1}	CH_2 stretching
	2850.59 cm^{-1}	CH_3 stretching
	1795.6 cm^{-1}	C=O stretching
	1711.05 cm^{-1}	C=O stretching
	1462.13 cm^{-1}	CH_2 bending
	1377.22 cm^{-1}	CH_3 bending
	1260.27 cm^{-1}	C-O stretching
Ethyl-Acetate fractions:	3438.09 cm^{-1}	OH
	2923.79 cm^{-1}	CH_2 stretching
Ethyl-Acetate 1	2852.81 cm^{-1}	CH_3 stretching
	1641.09cm^{-1}	Aromatic C=C
	1374.60cm^{-1}	CH_3 bending
	1281.05cm^{-1}	C-O stretching
	665.37cm^{-1} (broad)	N-H wag
Ethyl-Acetate:	3443.53cm^{-1} (broad)	OH stretching
	2923.7cm^{-1}	CH_2 stretching
	2852.93 cm^{-1}	CH_3 stretching
C. colocynthis 2 Fractions	Bands present	Functional groups assigned
	2076.26cm^{-1}	Aromatic Overtone
	1706.40cm^{-1}	C=O stretching
	1634.21cm^{-1}	Aromatic C=C stretching
	1462.41cm^{-1}	Aromatic C=C stretching
	1236cm^{-1}	C-O stretching

	663.02 (cm^{-1} broad)	N-H wag
Ethyl-Acetate:	3438.09cm^{-1}	
	2921.79cm^{-1}	CH_2 stretching
	2832.81cm^{-1}	CH_3 stretching
	1708.83cm^{-1}	C=O stretching
C. colocynthis 3	1641.09.cm^{-1}	Aromatic C=C
	1460.57cm^{-1}	
	1418.4cm^{-1}	CH_2 bending
	1281.05cm^{-1}	C-O stretching
	721.83cm^{-1}	Aromatic out of plane bending
	665.37cm^{-1}	N-H wagging
Butanol fractions:	Two fractions	
	3444.76cm^{-1}	OH stretching
Butanol: *C. colocynthis* 1	1706.4 cm^{-1}	C=O stretching
	1635.27cm^{-1}	, C=C stretching
	1459.88 cm^{-1}	Aromatic C=C stretching
	1280.7 cm^{-1}	C-O stretching
	719.63cm^{-1}	out of aromatic plane bending
	3426.12cm^{-1}	OH stretching
Butanol:	2921.31 cm^{-1}	CH_2 stretching
	2852.22 cm^{-1}	CH_3 stretching
C. colocynthis 2	1758.29 cm^{-1}	C=O
	1648 cm^{-1}	C=C
	1376.64 cm^{-1}	CH_3 bending
	1243.65 cm^{-1}	C-O

Discussion

In this study, phytochemicals recognized in the methanol extracts of *C. colocynthis* were largely alkaloids, saponins, reducing sugar, tannins, flavonoids, and phenolic compounds. This observation is consistent with finding from another investigation (Ahmad *et al*., 2018). These phytochemicals are known for their strong medicinal properties providing scientific evidence for the therapeutic use of this fruit in the management of ROS-induced diseases such as cancer (Yonbawi *et al*., 2021). Plant secondary metabolites including phenolics are recognised to play a vital role in antioxidant defense. Polyphenols or phenolic compounds, which are mostly tannins and flavonoids, are greatly regarded as powerful antioxidants, antibacterial, antiviral, anti-cancer and anti-inflammatory agents (Benariba *et al* 2013, Konappa *et al*., 2020). Yonbawi *et al.* (2021), demonstrated the anti-proliferative effect of this plant against HaCaT cells, as it showed a considerable decrease in cell growth. In another study, when an ointment prepared from methanolic extract of fruit pulp of *C. colocynthis* was applied to wound dressing on a rat model, a considerable wound contraction and an increase in hydroxyproline content of granulation tissue were observed (Gupta *et al*., 2018).

In this study, the profiling of the hexane fractions gave functional groups which revealed two components: reducing sugars and cardiac glycosides. The glycosides may have linkages at the -OH of the fatty acids. These results are in agreement with previous literature reports on fatty acids composition of *C. colocynthis* L. seed oil from different origin (Al-Hwaiti *et al*., 2021). The functional groups identified from FTIR assay revealed the presence of terpenoids and alkaloids. The butanol fraction, on the other hand, has two components, both of which contain phenolics. Steroids were found in higher concentration in the second component of the butanol fraction. In the same vein, Hatam *et al.* 1990 found two sterols in an oil extract of *C. colocynthis* fruits that were collected in the Basra region of Iraq. Plant sterols are particularly intriguing because of their antioxidant properties and potential as functional foods (Sabiri *et al*., 2019)

The in-vitro comparative analysis of the antioxidant potential of the fruit extract of *C. colocynthis* and ascorbic acid revealed that the plant extract had much stronger antioxidant activity than ascorbic acid. The high tannin and flavonoid contents of the fruit extracts could certainly be responsible for this. This result was however at variance with the study conducted by Ettebong *et al.* (2021) on the methanolic extract of C. lanatus seeds. These authors observed that ascorbic acid had the highest scavenging capacity over the seed extracts and fractions.

We observed that the nutritional composition of the fruit of *C. colocynthis* include high moisture content (81 %). This outcome was not unexpected, given that Cucurbitaceae plants are known for having large amounts of water in their fruits (Bhasin *et al*., 2020). Similar results were documented regarding the fruit extract of C. lanatus (Olayinka and Etejere 2018). In the present study, the total ash (9 %) and carbohydrate (7 %) contents of the fruits did not conform to those reported by QuratulAin *et al.* (2019)

Conclusion

The functional groups identified by FTIR support the secondary metabolites identified from the phytochemical screening. The plant extract displays high antioxidant activity in comparison to the standard (ascorbic acid) used in the in vitro antioxidant experiments. Due to this high antioxidant characteristics as well as nutritional features, *C. colocynthis* fruits may serve as natural materials for the synthesis of medications that could be effective in the treatment of diseases caused by reactive oxygen species. However, more research is needed to separate the key ingredients that are responsible for the antioxidant properties.

Acknowledgement The authors acknowledge the contribution of Dr. S. A. Adesida of the Department of Microbiology University of Lagos in proofreading the paper.

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