Evaluation of *Sorghum bicolor* (L.) Moench on the Reduction of Creatinine Levels and Its Antioxidant Properties *In vitro*

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(Received: April 2, 2022; Accepted: September 9, 2022; Published (Web): December 20, 2022)

ABSTRACT: The study investigated the effect of *Sorghum bicolor* (L.) Moench extract on the renal activity of both normal and ailing patients. Phytochemical analysis, antioxidant activity, vacuum liquid chromatography and TLC were carried out. The creatinine reduction ability was analyzed using Jaffe's method for clean-catch spot urine samples of normal, diabetic and hypertensive patients. *S. bicolor* showed strong antioxidant activity with an IC_{50} value of 52.17 µg/ml, compared to the standard vitamin C with an IC_{50} value of 40.59 µg/ml. Statistical analysis (ANOVA) result showed a significant difference between the patients' urine (rows) (p-value = 2.77×10^{-08}) and a significant difference between the dose treatments (columns) (p-value = 8.38×10^{-14}). Whilst different fractions of *S. bicolor* at different concentrations showed varying effects on the creatinine concentration, it reduced creatinine level in low doses and has antioxidant properties.

Key words: *Sorghum bicolor*, creatinine, antioxidant, reno-protective, kidney, VLC, *in-vitro*, chromatography, DPPH.

INTRODUCTION

Creatinine is a derivative of creatine phosphate breakdown in muscle, and is usually produced and excreted equally at quite a constant rate by the body under stable state.[1,2] The creatinine production rate per day is 10-15 mg/kg in children, 15-20 mg/kg in women and 20-25 mg/kg in men.[2] The normal range differ depending on the age group, body size, diet, and gender.[3] The creatinine concentration in urine decrease sharply after taking diuretics or drinking water and alcoholic beverages.[4] The utmost problem with using urinary creatinine to estimate body composition is the normal large daily variability of creatinine excretion within individual, although some people excrete uniform amounts of creatinine.[5] Monošík and Dragsted[6] in their study of dried urine swabs as a tool for monitoring metabolite excretion, measured creatinine level using a spot urine sample and stated that it reduces the problems associated with 24-h urine collection for both the scientist and the volunteers.[6] The quantity of both filtered and secreted creatinine expelled from the urine represents the difference between creatinine generation and its extra-renal excretion; the former is proportional to muscle mass but also affected by meat intake and the latter is known to increase in patients with severely reduced kidney function.[7]

In addition to using plants as food, specific plants make excellent natural medicines. Plants generally are really cleansing, healing, soothing and revitalizing as well as nourishing. Plants have an advantage in therapy, based on their long term use by humans as the main ingredients of traditional medicine. Around 100 plant species have contributed significantly to modern drugs.[8] Several medicinal plants including *Carica papaya* (Pawpaw), *Elaeis guineensis* (Oil palm), *Phoenix dactylifera* (Date), *Kigelia africana* (Sausage), *Anacardium occidentale* (Cashew) and *Mangifera indica* (Mango) have been

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Dhaka Univ. J. Pharm. Sci. **21**(2): 165-172, 2022 (December)
DOI: https://doi.org/10.3329/dujps.v21i2.63117

reported to have creatinine reducing effects and reno-protective potential.[9,10] Thus, this study aims to evaluate the preventive effect of *S. bicolor* extract on the renal activity of both normal and ailing patients.

Sorghum bicolor (Linn.) Pers. belongs to family Poaceae (Gramineae).[11] *S. bicolor* commonly called sorghum, Great millet, Guinea corn, Shattercane in English; Durra in Sweden; Jowar in India; Soro in Igbo; Okababa in Yoruba; Dawa in Hausa[12] is the fifth most important cereal crop in the world after wheat, rice, corn and barley.[11,13] The grain stalk, leaves, and leaf sheath contribute significantly in nutrition and medicine.[14] In most African countries, Sorghum is processed into food and beverages, which are important sources of nutrients. The leaf sheath black-purple color of *S. bicolor* is an indicator of the plant's potential health benefit in the management of sickle cell disease's anemia, joint pain, improving immune system, and heart, kidney and liver diseases.[14] *S. bicolor* leaf sheath play an important role in heart, immune system, kidney, and liver diseases.[13] Generally, *S. bicolor* is rich in minerals such as iron, sodium, and potassium[15] and being higher in protein and lower in fat constituent may be responsible for its hemopoietin ability.[16] It has been reported that *Sorghum* can be used as an anti-abortive, cyanogenetic, diuretic, emollient, demulcent, intoxicant and poison, and is also a folk remedy for cancer, flux epilepsy and stomach ache.[17] The root is used for malaria in Southern Rhodesia, the seed is used for the treatment of breast disease and diarrhea while the stem is employed for tubercular swellings' treatment.[14] *Sorghum* contains vitamins (D, E, K, thiamine, riboflavin and pyridoxine) and phenolic compounds which is a plant secondary metabolite that serve as antioxidant which are useful in the prevention of free radical mediated diseases.[18] The phytochemical screening of *Sorghum* indicated the presence of carbohydrates, phenolic acids, tannins, cardiac glycosides, phytates, lectins, flavonoids, stilbenes, glycosides, terpenoid, trypsin, chymotrypsin, amylase, polycosanols and phytosterols.[14] Kwon and Kim found thatethylacetate extracts from the stem of *S. bicolor* showed a strong free radical scavenging activity and used chromatography to isolate the antioxidants; as a result five major compounds were isolated from this fraction, to wit: methyl ferulate, methyl p-hydroxycinnamate, p-hydroxybenzaldehyde, tricin, and quercetin 3,4'-dimethyl ether.[19] Oladiji *et al.* studied the effects of oral administration of aqueous extract of *S. bicolor* stem bark to iron sufficient and iron deficient weaning rats, and it revealed that extract administration restored the anemic condition in the iron deficient group.[20] Nwinyi *et al.* in their study on the toxicity profile of methanol leaf base extract of *S. bicolor* in rats found that the extract produced no significant effect on total bilirubin, total protein, creatinine, urea and cholesterol levels.[21] Akande *et al.* used aqueous extract of *S. bicolor* leaf sheath on rats and also reported that compared with the control rats, the total protein concentration of the tested rats was not significantly altered by the extract.[22] The purpose of studying the effect of *S. bicolor* on the level of creatinine concentration is for its prospective use as both a herbal and nutritional remedy for maintaining and preventing renal malfunction.

METERIALS AND METHODS

Plant collection and preparation. *S. bicolor* was collected from a Sorghum farm upon harvest in Ibadan, Oyo state, Nigeria. A voucher specimen was deposited in Forest Herbarium, Ibadan, Nigeria with voucher numbers FHI 113027. The stems of *S. bicolor* were cut into bits and dried in the oven for 72h and pulverized using Christy and Norris 8" laboratory milling machine, after which it was weighed and kept in a cool and dry place for extraction.

Extraction and filtration. Finely ground *S. bicolor* stem was macerated in absolute ethanol for 11 days. The maceration was done by soaking the plants in airtight amber glass bottles and vigorous shaking at intervals. After maceration, a muslin cloth was used to filter and obtain the extracted filtrates. The filtrates were transferred into a rotary evaporator to concentrate and then poured into a crucible and put in a water bath to dry, leaving the concentrated

extracts. The dried extract was transferred into McCartney bottles and stored in the refrigerator.

Collection of urine samples. Urine samples were collected from three female adults, one of each category among normal volunteers, diabetic patients and hypertensive patients. The urine were collected into sample bottles, properly labeled, kept in cellophane bags and stored in the refrigerator to preserve it, prior to when needed. All information was handled with uttermost confidentiality and the research subjects reserve the right to privacy of non-disclosure of their details. A written informed consent was obtained from the subjects. The research received ethical approval from College of Medicine, University of Lagos health research ethics committee with the CMULHREC number CMUL/HREC/02/20/ 717 on 28th February 2020.

Phytochemical screening. The ethanol extract of leaf of *S. bicolor* was screened for the presence of sugars, alkaloids, coumarins, glycosides, phenols, phlobatanins, proteins, quinolones, saponins, steroids, terpenoids, volatile oils, cardiac glycosides and tannins by method described in relevant literature.[23,24]

DPPH radical scavenging activity assay. The free radical scavenging activity of the extracts, based on the scavenging of the stable 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical was evaluated. About 0.06 g of extract was mixed in methanol to make a stock (triplicate for each) solution of concentration 1000 µg/ml. Different concentrations of 20, 40, 60, 80 and 100 µg/ml were made from the stock (triplicate for each) solution and 2 ml of reagent solution (0.004g of DPPH in 300 ml methanol) was added to 3 ml of the aliquot of extracts in methanol. The blank contained only methanol and was used as the control. The mixture was vigorously shaken and left to stand at room temperature in a dark cupboard. After standing for 30 minutes, the decrease in absorbance of test mixtures was read at 517 nm.[25,26] The scavenging effect was calculated using the following expression:

$$\% \text{ inhibition} = \frac{A0 - A1}{A0} \text{ X } 100\%$$

where, A^0 is the absorption of the blank sample and A^1 is the absorption of the extract. IC_{50} was determined.[25, 26]

Chromatography. The crude extract adsorbed with silica gel at concentration 1:5 (W/W) was subjected to vacuum liquid chromatography (VLC) on silica gel dry packed column[27]using the solvents n-hexane, ethyl acetate and methanol in a gradient ratio of 100%, 75%:25%, 50%:50%, and 25%:75% (V/V) which yielded nine fractions. The fractions were collected in beakers and left to evaporate. The different fractions were labeled A, B, C, D, E, F, G, H and I to represent the different solvent gradient ratios. A methanol solution of small quantities of the fractions were made and analyzed on silica gel coated aluminum TLC plate developed in chloroform: methanol (0.5:9.5) utilizing 366 nm UV radiation to visualize and calculate the R_f (retardation factor) value of the components of the fractions.[28]

Creatinine test. This test was performed using two packs of Agappe creatinine 4×50 ml kit consisting of creatinine standard, creatinine base and creatinine dye. The standard was prepared according to the product description.

Determination of performed creatinine. 0.5 ml of urine sample was taken into a 50 ml glass-stopper cylinder which was made up to mark with distilled water (1:100 dilution factor). 3ml was taken from the diluted sample into a test tube and 1ml creatinine base (R1) was added, followed by 1ml of creatinine dye (R2). The test tube was shaken and left to stand for 2 minutes, there was a color change and the intensity of color was determined in the UV spectrophotometer at 517 nm. This procedure was repeated for all urine samples collected from the various categories of patients and the readings were carried out in triplicate. The amount of creatinine in g/l was determined using the formula:

$$\text{Creatinine concentration} = \frac{\text{Absorbance of test X standard concentration X dilution factor}}{\text{Absorbance of standard X 100 (convertion factor from} \frac{\text{mg}}{\text{dl}})}$$

Determination of the effect of extracts and fractions on creatinine in urine samples collected from patients. 1%, 5% and 10% concentrations of the extract and fractions were prepared (10, 50, 100 µg/ml). The absorbance and creatinine concentration were determined similar to that of control group. Here 1 ml of the extracts or fractions added (doses mentioned) singly into a test-tube containing 3 ml of diluted urine samples before measuring the absorbance. The procedure was repeated for all the urine samples collected at different concentrations of the extracts and fractions, and readings were taken in triplicate.

RESULTS AND DISCUSSION

S. bicolor was evaluated *in-vitro* for potential use in the reduction of creatinine level and its antioxidant activity. The three female adults used were interviewed for drug use and diet plans, but only the hypertensive and diabetic patients agreed to be on drugs and specialized diet.

Phytochemical analysis. The preliminary phytochemical screening of the plant extract showed (Table 1) presence of some phytochemicals capable of bestowing health benefits[29] viz; alkaloids, carbohydrates, courmarins, flavonoids, glycosides, phenols, proteins, reducing sugars, saponins, tannins, terpenoids and volatile oils.

Table 1. Phytochemical screening tests of *S. bicolor*.

Phytochemical	*S. bicolor*
Alkaloids	+
Carbohydrates	+
Cardiac glycosides	-
Courmarins	+
Flavonoids	+
Glycosides	+
Phenols	+
Phlobatanins	-
Proteins	+
Quinones	-
Reducing sugars	+
Saponins	+
Steroids	-
Tannins	+
Terpenoids	+
Volatile oils	+

+ means positive/present, - means negative/absent.

The absence of cardiac glycoside and the presence of alkaloid and saponin in *S. bicolor* opposes the report of Salawu *et. al.* 2014 on the presence of cardiac glycosides, flavonoids, glycosides, and tannins and the absence of alkaloids and saponins.[14]

Antioxidant activity. The DPPH scavenging activity of the methanol extract of *S. bicolor* at concentrations 20 to 100 µg/ml was determined (Table 2) using vitamin C as standard. Here, vitamin C showed highest percent inhibition of 90.70%, 92.90% and 92.60% at 60, 80 and 100 µg/ml concentration while *S. bicolor* showed highest percent inhibition of 75.9% and 73.99% at 40 and 60 µg/ml, respectively. The minimum inhibition was obtained at 20 µg/ml for both vitamin C and *S. bicolor*. The half-maximal inhibitory concentration (IC_{50}) value, defined as the concentration required for 50% inhibition, of *S. bicolor* is 52.17 µg/ml which is higher than that of the standard vitamin C at 40.59 µg/ml showing strong antioxidant activity.

Compared to the standard vitamin C, *S. bicolor* extract showed a significant DPPH radical scavenging activity. This result is in close agreement with the report of Kwon and Kim on the promising antioxidant potential of ethyl acetate fraction from the stem of *S. bicolor*.[19] The IC_{50} value obtained in this study is similar to the value 53.83 µg/ml obtained for the methanol whole grain extract by Mami-Soualem.[30] According to Dennis and Witting, antioxidants are protective against oxidation, inflammation and kidney damage and several dietary plant polyphenols and flavonoids including curcumin, which are shown to be present in the extract, are efficacious in rhabdomyolysis and ischemic acute kidney injury in animal models.[31] Since increased oxidative stress is involved in the mechanisms of many forms of renal injury, it is safe to say that the antioxidant property of these plants is somewhat involved in their creatinine reduction potential.[32]

Table 2. DPPH scavenging activity with IC_{50} values.

Concentration (µg/ml)		20	40	60	80	100	IC_{50}
% Inhibition	Vit C	67.50	90.40	90.70	92.90	92.60	40.59
	S. bicolor	61.60	75.90	73.99	69.97	67.80	52.17

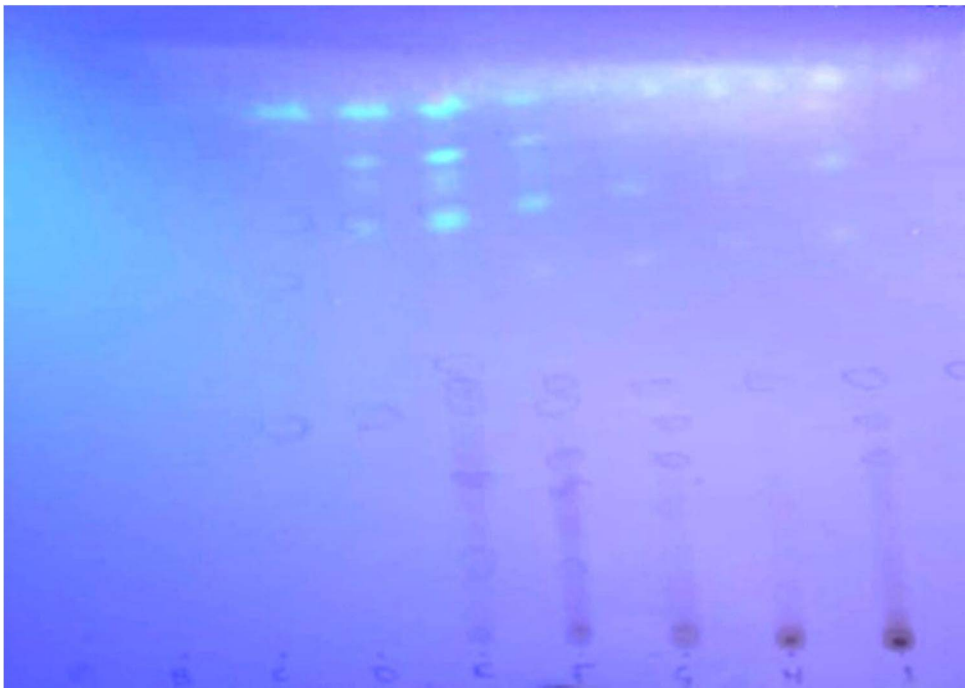

Figure 1. *Sorghum bicolor* fractions developed on TLC plate using the solvent system chloroform: methanol (0.5:9.5). A 100% n-hexane, B 75% n-hexane : 25% ethyl acetate, C 50% n-hexane : 50% ethyl acetate, D 25% n-hexane : 75% ethyl acetate, E 100% ethyl acetate, F 75% ethyl acetate : 25% methanol, G 50% ethyl acetate : 50% methanol, H 25% ethyl acetate : 75% methanol and I 100% methanol.

The identification of bioactive compounds. The extract seemed to have higher affinity to the more polar compound. The chromatography results (Table 3 & Figure 1) showed the presence of different compounds.

Determination of reduction in the creatinine concentration. In the results of normal volunteers, the addition of *S. bicolor* treatment showed a decrease in the creatinine concentrations (Table 5). Although the initial drastic reduction in creatinine concentration, which was noticed in treatments of crude extract 10 (0.25), crude extract 50 (0.36), crude extract 100 (0.71), A10 (0.23), A50 (0.28) and A100 (0.83), it showed a sudden increase from treatment B10 (3.41). However, this concentration was still lower than the control group. There was marked increase in the creatinine concentrations at treatments F100 (5.53), G100 (7.92), H50 (5.27), H100 (7.05), I100 (5.29). In the results of hypertensive patients (Table 5) the addition of *S. bicolor* treatments showed an increase in creatinine concentration compared to the control, especially at G100 (5.43), H100 (4.05). But at treatments A10 (0.31), A50 (0.22), A100 (0.37) and B10 (0.35) there was a reduction in the creatinine concentrations. In the results of diabetic patients (Table 5), the addition of *S. bicolor* treatments showed an increase in the creatinine concentration except in treatments D10 (1.88), D50 (1.56), E10 (1.72), F10 (1.98), G10 (1.10) and H10 (1.04) where a marked decrease was reported. From the ANOVA table (Table 6), it can be seen on the row that the p-value = 2.77×10^{-08} was

less than 0.05 significant level and the F-statistics = 5.221244 was larger than F-critical =1.649141; therefore, there is significant difference between the rows. It can be seen on the column that the p-value = 8.38×10^{-14} was less than 0.05 significant level and the F-statistics = 51.84981 was larger than F-critical = 3.150411; therefore, there is significant difference between the columns. This means that both the urine types and dose treatments of crude extract and its fractions has a significant effect on the results, which could be either negative or positive.

Table 3. R_f values from TLC of *S. bicolor*.

Fractions	R_f values
A	No movement observed
B	No movement observed
C	0.38, 0.66, 0.75, 0.86, 0.94
D	0.39, 0.73, 0.80, 0.84, 0.94
E	0.14, 0.29, 0.41, 0.44, 0.49, 0.74, 0.81, 0.84, 0.94
F	0.13, 0.25, 0.33, 0.41, 0.46, 0.66, 0.78, 0.88, 0.95
G	0.10, 0.31, 0.38, 0.45, 0.68, 0.80
H	0.46, 0.71, 0.83, 0.96
I	0.09, 0.33, 0.39, 0.47, 0.72, 0.84, 0.97

Table 4. VLC fractionation of *S. bicolor*.

Samples	Solvent gradient	Color
A	100% n-hexane	Colorless
B	75% n-hexane : 25% ethyl acetate	Colorless
C	50% n-hexane : 50% ethyl acetate	Pink tint
D	25% n-hexane : 75% ethyl acetate	Light pink
E	100% ethyl acetate	Pink
F	75% ethyl acetate : 25% methanol	Red
G	50% ethyl acetate : 50% methanol	Red
H	25% ethyl acetate : 75% methanol	Blood red
I	100% methanol	Dark red

The extracts at different doses showed distinct effects on the creatinine concentration of the patients' urine, which could be attributed to the presence or absence of bioactive compounds available in the extract and its fractions due to variation in solubility of these compounds to solvents during the VLC fractionation (Table 4). There was creatinine reduction capacity at specific doses of all treatments, but the highest reduction was noticed in the lowest dose of fraction A in normal patients. The controls are within the normal range noted in the product description except for the high concentration noticed in the normal volunteer which could be attributed to the fact that diet, drugs, alcohol, water and exercise can affect creatinine level.[3,4,33] Salawu and Salimon reported an increase in creatinine of iron deficient and iron sufficient rat fed different concentrations of aqueous

Table 5. Creatinine concentration at different treatments using *S. bicolor*.

Sample	Dose	Normal urine (g/l)	Hypertensive urine (g/l)	Diabetic urine (g/l)
Control		4.75±0.038	0.49±0.020	2.01±0.016
Crude extract	10	0.25±0.005	0.54±0.036	2.04±0.044
	50	0.36±0.002	0.66±0.031	2.35±0.009
	100	0.71±0.019	1.18±0.005	2.87±0.027
A	10	0.23±0.001	0.31±0.004	2.47±0.075
	50	0.28±0.004	0.22±0.002	2.55±0.035
	100	0.83±0.104	0.37±0.042	2.96±0.008
B	10	3.41±0.106	0.35±0.007	2.58±0.016
	50	3.67±0.067	0.52±0.018	2.63±0.045
	100	4.73±0.055	0.83±0.056	3.24±0.073
C	10	3.60±0.116	0.58±0.022	2.04±0.062
	50	4.18±0.013	0.89±0.015	2.16±0.027
	100	3.96±0.120	1.23±0.007	2.71±0.062
D	10	2.64±0.239	0.60±0.011	1.88±0.037
	50	4.19±0.048	0.98±0.023	1.56±0.017
	100	4.78±0.003	1.29±0.002	2.10±0.013
E	10	3.22±0.140	0.63±0.018	1.72±0.024
	50	3.47±0.115	0.70±0.009	2.27±0.084
	100	4.48±0.006	1.15±0.017	3.13±0.002
F	10	3.62±0.036	0.50±0.022	1.98±0.011
	50	4.21±0.137	1.40±0.011	2.56±0.011
	100	5.53±0.094	3.43±0.014	3.36±0.072
G	10	4.10±0.042	1.10±0.024	1.55±0.001
	50	3.92±0.266	2.57±0.016	3.26±0.014
	100	7.92±0.002	5.43±0.026	5.80±0.045
H	10	4.06±0.130	1.04±0.013	1.41±0.053
	50	5.27±0.098	2.10±0.010	2.80±0.083
	100	7.05±0.107	4.05±0.031	4.81±0.022
I	10	3.61±0.116	1.03±0.013	2.48±0.051
	50	4.48±0.099	1.48±0.001	3.19±0.002
	100	5.29±0.156	2.85±0.011of	3.96±0.118

Table 6. **Two-way ANOVA table of *S. bicolor* treatments (from table 5).**

Source	(SS)	(Df)	(MS)	F-statistics	p-value	F-critical
Rows (urine)	128.4261	30	4.28087	5.221244	2.77E-08	1.649141
Columns (treatment)	85.02277	2	42.51138	51.84981	8.38E-14	3.150411
Error	49.19368	60	0.819895			
Total	262.6426	92				

extract of *S. bicolor*, which agrees with the results of our study that indicates *S. bicolor* increases creatinine level at high doses.[11] It can be hypothesized that the mechanism of reducing the creatinine level by *S. bicolor* extract is due to creatinine degradation. Creatinine can be degraded back to creatine and may proceed further to creatol, methylguanidine, urea, sarcosine, methylamine, glyoxylate and glycolate.[34] The antioxidant compounds contained in the plant could also be responsible for the reduced creatinine level.

In conclusion, all treatments of *S. bicolor* in low doses can be used in the reduction of creatinine level in normal patients but only fractions D, E, F, G, and H in low doses can be used in diabetic patients to reduce creatinine level, and in hypertensive patients, all doses of fraction A and low dose of faction B can be used in the reduction of creatinine level. It can also be deduced from the study that *S. bicolor* extracts have a significant DPPH radical scavenging capacity, in essence it contains antioxidant compounds. Further studies will be needed to clarify the structural identity of the active fractions.

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