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(RESEARCH ARTICLE)

Phytochemical screening and antibacterial activities of *Sorghum bicolor* leaves derived from *in vitro* culture

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Abstract

Various research works recognized highly biological activities of sorghum vegetative portions which indicated the presence of bioactive compounds in their extracts. Considering environmental effects on the accumulation of secondary metabolites, this work aims to evaluate the antibacterial activity of sorghum using *in vitro* induced leaves as source for extract. *In vitro* shoot explants of sorghum used subcultured on Murashige and Skoog medium (MS) supplemented with 6-Benzyl adenine (BA) or Indole-3-butyric acid (IBA) at different concentrations (0.0–2.0 mg/L). The leaves induced *in vitro* were collected dried then macerated in ethanol for 4 hours. Phytochemical composition of the sorghum leaves extract was assessed using standard procedures. The crude extracts were evaluated for antibacterial activity using the agar well diffusion method. The significantly (P>0.05) maximum shoot length (5.7 cm) and the number of leaves (7.9 leaves) were obtained on MS medium supplemented with 2.0 mg/L IBA. The phytochemical composition of the leaves extract showed the presence of bioactive constituents including alkaloids, flavonoids, tannins, saponins and steroids and triterpenes. All the concentrations of the sorghum leaves extract showed variable antimicrobial activity against the studied bacteria strains with the strongest inhibitory effect reported (19.0 mm) against *B. subtilis* at the concentration of 100 mg/L. Our findings demonstrated that the *in vitro* leaves extract of sorghum possess a remarkable antibacterial activity. More research is needed on the characterization of bioactive ingredients of *in vitro* induced sorghum plants and their biological activities.

Keywords: Antimicrobial; Sorghum; *In vitro* leaves; Phytoconstitutes

1. Introduction

Sorghum bicolor (L.) Moench subsp. *bicolor* (Family: Poaceae) is one of the most important cereal crops which came the fifth after rice, wheat, maize and barley in the world and main food grain production in Africa, Central America, and southern Asia [1]. However, sorghum is more tolerant to diseases, pests, drought and poor soils. Sorghum is the prevalent crop in Sudan covering an area of 9.8 million hectares which represented more than 60% of the total cultivated cereals area, with a production of about 4.9 million tons contributing to 9.1% of total world production in 2018 [2].

Sorghum used mainly for grain production in addition to cultivation for syrup production, feeding, and biofuel production. Other important uses, especially from leaves, sheath, stalks, glumes, and root were also available including

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food additives and folk pharmaceuticals. Red or purple pigments are largely extracted from the sheath and used as food colourants such as cheese and porridges, and dyes for leather, textile, and wood in numerous countries of Africa and Asia [3-5]. Sorghum extracts is reported to have antiabortion, cyanogenic, demulcent, diuretic, emollient, intoxicant, and toxic properties [6]. Various traditional remedies were prepared from sorghum to treat cancer, breast disease, malaria, tubercular swellings, eczema, dysentery, diarrhea, stomach ache, helminths, bone pains, epilepsy, sickle cell disorder, anemia and as a blood builder [6-8].

The therapeutic roles reported on sorghum are results of anti-inflammatory, anti-carcinogenic, antibiotic, antifungal, antiviral, hepatoprotective, anti-ulcer, anti-neoplastic, cholesterol-lowering and digestibility slowing properties [8-11]. Such pharmaceutical functions are allied to the phytochemical contents of the plant such as phytosterols, policosanols, saponins, carotenoids and phenolic compounds, including flavonoids, tannins, and anthocyanins [12]. Different important flavonoids and phenolic acids were identified in the leaves extract of sorghum including gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, rutin, isoquercitrin, quercitrin, quercetin, and kaempferol [13]. The pigmented shell contains a high concentration of phenolic compounds including phenolic acids, 3-deoxyanthocyanins and condensed tannins [11]. Almost all classes of phenolic compounds are found in sorghum in the form of phenolic acids, flavonoids and condensed tannins [14]. The total phenol concentrations estimated to be 1.1–1.5% of root dry weight and 1.1–2.2% of aerial parts dry weight of the sorghum grown in Senegal [15]. The secondary metabolites reported on sorghum are integral cellular structural or components of plant cell walls and the compositions depend on the sorghum genotype and the environmental factors during crop growth [16]. Genetic factors controlling seed colour and feature, and also secondary plant colour which affects the appearance of leaf, stalk, sheath, glume, and kernel [11]. Environmental factors including pests and diseases that induce stress on sorghum trigger phytoalexins such as flavonoids as defense response, and plant sterols and policosanols are constituents of wax [9,17] vital in alleviating evaporation. Regarding the constitutive defense benzoxazinoids, nitrogen-containing metabolites, are the predominant phytoanticipins in allelopathy mechanism, and against insects and microorganisms in sorghum [17]. *In vitro* plant tissue culture technique can be used to produce a plant with valuable secondary products that may not be accumulated in response to environmental conditions.

Due to the extraordinary beneficial phytochemicals content of sorghum vegetative parts, various research on the biological activities was done. Such studies include antimicrobial activity of stem extract [3], *in vitro* anti-inflammatory and immune-modulating properties of leaf sheath [18] and various *in vivo* studies on rats using sorghum leaves, leaf base, and leaf sheath extracts proved antisickling [7], enhance the hematological parameters [19], anti-anaemic stem bark [20], for anti-nociceptive and anti-inflammatory activities [21] and hepatotoxicity and oxidative stress inhibitor [8]. However, more research is needed on biological activities of vegetative parts of sorghum.

To the best of our knowledge, there are no published research work on the antimicrobial activity of *in vitro* derived plant parts of sorghum. Therefore the purpose of this study was to validate the phytochemical and antimicrobial activity of sorghum using *in vitro* induced leaves.

2. Material and methods

2.1. *In vitro* culture of sorghum

In vitro shoot (1.5-2.0 cm long with 2-3 leaves) of *Sorghum bicolor* cultivar Butana used as explants. The shoots were already maintained on Murashige and Skoog (MS) medium [22] fortified with 1.0 mg/L 6-Benzyl adenine (BA). Shoots explants were subcultured on MS medium supplemented with different concentrations (0.0, 1.0, 2.0 mg/L) of BA or Indole-3-butyric acid (IBA). MS media was augmented with 3% sucrose and the pH was adjusted to 5.8 before autoclaving at 121°C (1.1 Kg/cm^3) for 15 min. All cultures were incubated at 25 ± 2°C under a photoperiod of 16 h light/8 h dark. Data on shoot induction, shoot length, number of leaves, and rooting per shoot were collected after 4 weeks of culture.

2.2. Plant material and extract preparation

Fully devolved leaves were collected from *in vitro* raised shoots of sorghum culture, air-dried and crunched then stored in a tight container till use. Powdered leaves (100 g) were extracted successively in ethanol with aid of magnetic stirring apparatus for 4 hours at room temperature. The ethanol extract was filtrated with filter paper (Whatman No. 1) then evaporated and air-dried and the residue, after final weight obtained, was kept at 4 °C until use.

2.3. Phytochemical screening

Phytochemical screening for the active materials was carried out for extracts using the methods described with some modification.

The presence of alkaloids in extracts is tested by using Wagner's reagent [23]. That, 2 mL of Wagner's reagent is added to 2 mL of extracts. The formation of reddish-brown precipitate indicated the presence of alkaloids.

The test for steroids was done according to the method with modifications [24]. Hence, 1 mL of extract was taken in a test tube and dissolved with 10 mL chloroform, then an equal volume of concentrated H_2SO_4 was added to the test tube by sides. The upper layer in the test tube appears red and the sulphuric acid layer showed yellow with green fluorescence, which indicated the presence of steroids.

Test for flavonoids in plant material by taking 2 mL of the extract in a test tube and 2-3 drops of dilute NaOH was added [24]. An intense yellow color has appeared in the test tube. The solution became colorless when a few drops of dilute H_2SO_4 were added confirming the presence of flavonoids.

Saponins were tested by taking 2 g of the powdered sample and boiled in 20 mL of distilled water in a water bath then filtered [25]. To the filtered sample (10 mL), about 5 mL distilled water was added, shaken vigorously and observed for a stable persistent frothing for 25 min.

Test for tannins was done with some modifications [25]. From the dried powdered sample, 0.5 g was boiled in 20 mL water in a test tube and then filtered. One milliliter of 0.1% Ferric Chloride (0.01 Mol/dm^3) was added to 2 mL of extract. Brownish green colorations were indicated the presence of tannins distilled water in a water bath and filtered. To the filtered sample (10 mL), about 5 mL distilled water was added, shaken vigorously and observed for a stable persistent frothing for 25 min.

2.4. Antimicrobial activity

Bacterial strains of two gram-positive; *Bacillus subtilis* and *Staphylococcus aureus* and two gram-negative; *Escherichia coli*, *Pseudomonas argenosa* were used to study the antibacterial activity of sorghum leaves extract. The paper disc diffusion method was used for the antibacterial screen and performed by using Mueller Hinton agar. The experiment was carried out according to the National Committee for Clinical Laboratory Standards guidelines [26]. The bacterial suspension was diluted with a sterile physiological solution to 108 CFU/mL (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on the surface of MHA and the inoculum was allowed to dry for 5 min. Sterilized filter paper discs (Whatman No. 1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µL of sorghum extract solution. Extract powder dissolved in DMSO was applied in four concentrations 12.5–100 mg/L. Negative control was also prepared in DMSO. The inoculated plates were incubated at 37°C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured and compared with reference antibiotic discs.

2.5. Statistical analysis

Every experiment was repeated three times. The obtained data were subjected to analysis of variance (ANOVA). The mean separation was analyzed using Duncan's new multiple range test at P-value <0.05. All the values were expressed as mean ± standard errors.

3. Results and discussion

3.1. *In vitro* culture of sorghum

The presence of IBA 2.0 mg/L enhanced noticeably the growth of the shoots (Table 1) with significantly (P>0.05) the longest shoots (5.7 cm) and the highest number of leaves (7.9 leaves). Moreover, some of the shoots were induced roots affected by the concentration of IBA. Addition of IBA at lower concentration (1.0 mg/L) favorite rooting of shoot compared to other plant growth regulators (PGRs) treatments (Table 1). Improving shoot growth and rooting of *in vitro* sorghum shoots by using IBA or auxins have been identified by several authors [27]. The presence of BA deteriorated shoot length (to 1.9 cm at 2.0 mg/L BA) compared to the control (2.0 cm) considering the initial shoot explant length (average 1.7 cm). A similar negative effect of increasing concentration of BA on shoot length of sorghum has been reported [28].

Table 1 Effect of IBA and BA on shoot length, number of leaves, the presence of multiple shoots and the presence of secondary roots after 4 weeks of culture**.**

PGRs (mg/L)	Shoot length (cm)	Number of leaves	Multiple shoots*	Rooting*
Control	2.0 ± 0.1c	3.0 ± 0.0d	-	-
IBA 1	3.15 ± 0.3b	5.62 ± 0.5b	-	++
IBA 2	5.69 ± 0.4a	7.85 ± 0.5a	+	+
BA 1	1.73 ± 0.1c	3.62 ± 0.2cd	++	-
BA 2	1.88 ± 0.4c	4.23 ± 0.3c	++	-

PGR: plant growth regulator; IBA: Indole-3-butyric acid; BA: 6-Benzyladenine; *relative frequency index: –, +, ++ = absent, low, high. Values represent means ± standard errors. *Different* letters indicated statistically significant *differences* between means according to Duncan's new multiple range test (P<0.05).

3.2. Phytochemical screening of sorghum leaves

The results of qualitative phytochemical screening of ethanol extract of sorghum leaves indicated the presence of alkaloids, tannins, flavonoids, saponins, sterol, and triterpenes (Table 2). Likewise, the presence of alkaloids, flavonoids, saponins, and terpenoids has been detected in different sorghum aerial parts such as aqueous extract of leaves [19], and aqueous extract of stalk/stem in which terpenoids was not detected [29]. In contrast, alkaloid, and steroids were not detected in both methanol and aqueous extracts of sorghum leaves [13].

Table 2 Phytochemical screening of *Sorghum bicolor in vitro* leaves extract**.**

Phytochemical	Presence index
Alkaloids	+
Flavonoids	+
Steroids and triterpenes	+
Tannins	+
Saponins	+

The leaves and sheath of sorghum had higher phenols contents, up to 600 times more than their respective grains, with *in vitro* antioxidant properties than commonly seen in grains [4]. The phenols in sorghums fall under two major categories; phenolic acids (benzoic or cinnamic acid derivatives) and flavonoids (tannins and anthocyanins) [8]. Various flavonoids were identified in sorghum include the flavanones eriodictyol, naringenin, and eriodictyol glucoside, the flavone apigenin, and the 3-deoxyanthocyanins luteolinidin and apigeninidin [11].

3.3. Antimicrobial activity of sorghum leaves

All the concentrations of the sorghum leaves extract showed high antimicrobial activity against the four studied bacteria strains (*S. aureus, B. subtilis, P. aeruginosa and E. coli*) when compared to standard drugs ciprofloxacin (Table 2). Obviously, the inhibitory effect of the extract increased significantly ($P \leq 0.05$) with increasing the concentration. The strongest inhibitory effect reported (19.0 mm) was against *B. subtilis* at 100 mg/L leaves extract. The minimum inhibitory zone recorded (with the concentration 100 mg/L) was 17.5 mm against *P. aeruginosa***.**

The crude leaves extract of sorghum was slightly more effective against gram-positive bacteria compared to gram-negative strains. Variations in response of the gram-positive bacteria and gram-negative strains to the different extracts of sorghum aerial parts have been reported by several authors. For example, the crude extracts of sorghum leaf sheath demonstrated activity against *B. subtilis* [30]. In contrast, sorghum crude stem extract showed high inhibition against *P. aeruginosa* (gram-negative) compared to *B. subtilis* (gram-positive) [3]. On the other hand, extracts of dye sorghum leaf sheaths found have no anti-bacterial properties on *E. coli* tested in a nutrient-rich traditional West African cheese [5].

Table 3 Antibacterial activity of sorghum leaves extract**.**

Antibiotic	Dose (mg/L)	Inhibition zone (mm)			
		Gram-positive bacteria		Gram-negative bacteria	
		B. s	***S. a***	***P. a***	***E. c***
Sorghum extract	0	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}	0.0 ± 0.0^{f}	0.0 ± 0.0^{e}
	12.5	10.2 ± 0.1^{e}	11.0 ± 1.0^{d}	10.0 ± 0.1^{e}	11.1 ± 1.0^{d}
	25	14.1 ± 0.1^{d}	12.1 ± 1.0^{d}	13.2 ± 1.2^{d}	14.0 ± 0.1^{c}
	50	16.0 ± 0.1^{c}	16.0 ± 0.1^{c}	15.0 ± 0.1^{c}	17.0 ± 1.0^{b}
	100	19.0 ± 1.0^{b}	18.0 ± 1.0^{b}	17.5 ± 0.1^{b}	18.0 ± 1.0^{b}
Ciprrofloxacin	100	31.0 ± 0.01^{a}	30.0 ± 1.0^{a}	29.0 ± 1.0^{a}	30.0 ± 0.1^{a}

B.s: *Bacillus subtilis*; *S. a*: *Staphylococcus aureus*; *P. a*: *Pseudomonas aeruginosa*; *E. c*: *Escherichia coli.* Values represent mean ± standard errors. *Different* letters indicated statistically significant *differences* between means according to Duncan's new multiple range test (P<0.05).

However, similar trends of exerting higher activity against gram-positive bacteria compared to gram-negative strains have been also reported on seed extracts of sorghum. Sorghum seeds extract exhibited moderate to high activity against *B. subtilis* and *S. aureus* compared to the studied gram-negative bacteria [31]. Also, the condensed tannin-free sorghum crude phenolic extract was found effective only against *B. cereus* (gram-positive), while none of the tested types of bran sorghum phenolic extracts had inhibitory effects on *E. coli* (gram-negative) [32]. Moreover, Saponins extract of sorghum seed inhibited the growth of the *S. aureus* indicated that the extract has an inhibitory effect on gram-positive organisms but not on gram-negative organisms [10]. Also, tannin extract of sorghum seed showed an inhibition effect on *S. aureus* growth higher than that on *E. coli* [33]. In contrast, all sorghum seed extracts and fractions showed a strong inhibitory effect against *E. coli*, and among all the studied four cultivars only one showed an inhibitory effect against *B. subtilis* [34]. Likewise, none of the methanolic extracts from pigmented grains of ten sorghum lines showed antibacterial activity against *B. subtilis* while nine of them strongly inhibited the growth of *E. coli* [35].

These variations in antibacterial activity between gram-positive and gram-negative bacteria are mainly due to variances in cell wall structures of the bacteria gram-type. There is a general rule that secondary metabolites present in plant extracts can inhibit gram-positive bacteria more than gram-negative bacteria [36]. For instance, the cell wall of gram-negative bacteria is surrounded by an outer additional lipopolysaccharide membrane, which functions as a hydrophilic surface that prevents permeability of hydrophobic compounds such as tannins and other many plant extracts [31, 36, 37].

The observed dissimilarity in the antimicrobial activities of sorghum extracts might be attributed to the plant genotype or type of solvent used for extraction which produces different bioactive substances and composition, among others. For example, various studies showed that flavonoid levels and composition were affected by the sorghum genotype [11]. Likewise, great variation in tannin content was found between three Sudanese sorghum cultivars [33]. Different solvent types produced different phytochemical compounds that exert different antimicrobial activity through different mechanisms. That, phytochemical analysis of sorghum leaves indicated the absence of saponin and tannin aqueous extract while were found in the methanol extract [13]. The phenolic compounds are toxic to bacterial cells and can inhibit bacteria growth by several actions such as modifying the permeability of cell membranes, changes in various intracellular functions induced by hydrogen binding to enzymes or by the modification of the cell wall rigidity with integrity losses due to different interactions with the cell membrane [38]. In the case of gram-positive bacteria, phenolic compounds intracellular pH modification, as well as interference with the energy (ATP) generating system, were reported [39]. The other secondary metabolites such as saponins are also contributed to the antimicrobial activity of sorghum and may act by damaging cell membranes causing leakage of cellular materials, ultimately leading to cell death [40].

4. Conclusion

Plant tissue cultures are an attractive alternative technique to the whole plant for the production of the high value of active substances. The results of this study pointed out that antimicrobial activities exhaled by *in vitro* leaves of *Sorghum bicolor* due to the presence of the bioactive ingredients. Further research is necessary to determine the identity of the therapeutic compound within this crude extract of the plant also to determine their full spectrums of efficiency.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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