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Antimicrobial activity of aqueous methanolic extracts of Algerian cultivars of sorghum

(*Sorghum bicolor* (L.) Moench)

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Abstract: *Certain phenolics have been recognized to possessing antibacterial and antifungal activities and high levels of flavonoids and tannins have been reported in several varieties of Sorghum bicolor (L.) Moench. Antimicrobial activity and phenolics contents were investigated in five Algerian sorghum seeds. AS20 sorghum extract showed the highest levels of: total phenolics (3214.46±263.64 mg/100 g), flavonoids (32.03±1.64 mg/100 g) and tannins (615.35±6.10 mg/g) contents; however, comparable flavonoids content was recorded in I27 extract. FZ40 and AS12 flavonoids contents were comparable. Screening for antimicrobial activity, carried out by the disc’s diffusion method revealed an antimicrobial potential of sorghum crude extracts against Gram-positive and Gram-negative bacteria and candida albicans yeast. Minimal inhibition concentration determined by microdilution method varied between 0.2 and 2 mg/ml. the lowest value was recorded with F11 and FZ40 extract against Streptococcus pneumoniae and F11 against Escherichia coli. Bacillus subtilis, Staphylococcus aureus ATCC6538 and MRSA strains showed sensitivity to all extracts. The results show these sorghums as a potential source of natural anti-streptococcal, anti-staphylococcal and anti-candida substances.*

Keywords: *Sorghum bicolor* (L.) Moench., phenolics, flavonoids, tannins, antimicrobial activity

Introduction

Sorghum flour is rich in phytochemical components with a potential to have important health-promoting properties [1-5]. Phenolic compounds in sorghum occur as phenolic acids, flavonoids and condensed tannins [6, 7]. Phenylpropane glycerides, dicaffeoyl spermidine and phenolamides are supplement classes of phenolic compounds which were, recently, found in sorghum grain [8]. The amount of phenolics in plants depends on both biological factors (genotype, organ and ontogeny) and edaphic and environmental (temperature, salinity, water stress and light intensity) conditions [9].

Sorghum cultivars in Algeria have the particularity to grow in hyper arid ecosystem where the maturity temperature is very high reaching a monthly mean of 45.2°C [10]. This high temperature and drought conditions are coupled with a high salinity of soil in “In Salah” province southern Algeria [11].

Tannin sorghum is sorghum lines with a pigmented testa. This is a layer immediately under the brand in the sorghum caryopsis. Tannins (proanthocyanidins) are deposited in this layer as the seed matures. The presence of testa deter entry of microorganisms into the kernel. Tannins reduce grain losses in the field caused of bird predation. They have been shown to promote human health because of their high antioxidant capacity and ability to fight obesity through reduced digestion [12].

Like most polyphenols, flavonoids have very potent antifungal activity [13, 14]. The antibacterial activity of flavonoids and tannins is recognized [15]. For example, cranberry (*Vaccinium macrocarpon* Aiton) has shown antifungal potential with epicatechin oligomers with DP>5 [16].

The antibacterial activity of certain phenolic acids like caffeic acid and benzoic acid and the antifungal activity of coumaric acid and ferulic acid have been reported in the literature [17, 18]. Actually, phenolic acids are believed to contribute to the defense of plants against pathogens [19]. Bacteria continue to develop resistance to antibiotics for multiple causes like the specific nature of the bacteria-antibiotics relationship, the failing usage of antibacterial agent and environmental factors. Consequently, a lot of infections are, at present, difficult to treat and the World Health Organization has named antibiotic resistance as one of the three most important public health threats of the 21st century [20]. Nevertheless, results of investigation on natural drugs, exhibiting antimicrobial activity, encourage researcher to further exploration to found available resources to limit the emergence of these organisms [13].

To date, there are limited reports on Algerian sorghum properties and their phenolic compounds. The objective of this work is to evaluate antimicrobial activity of aqueous methanolic extracts of sorghums cultivated in Saharan environment, related to their total polyphenols, flavonoids and tannin contents.

Material and Methods

Sorghum samples

Three sorghum cultivars with pigmented pericarp and testa, AS12, AS20, I27 and H40, were selected among 47 collected cultivars grown in “In Salah” region in Sothern Algeria, due to its agro-morphological characterization (Table 1). The grains were harvested in October 2010, cleaned then dried in free air and selected manually and sieved to remove impurities. Whole sorghum flour was obtained by grinding 100 g of cleaned grains of each cultivar in a laboratory impact mill (Cyclotec TM 1093 Sample Mill) and sieved through a 0.850 mm mesh screen [53].

Table 1. Some agro-morphological characteristics of sorghum cultivars, AS12, AS20, I27 and H40

Cultivars	GC/PC ^[a]	TP ^[b]	SC ^[c]	PC ^[d]	TGW ^[e]	RC ^[f]
F11	-/-	-	white	+	48,46	nd
AS12	+/+	+	Red	++	30.06	Durra-caudatum
AS20	+/+	+	Brown	-	14.83	Bicolor
I27	-/+	+	Brown	+	30.17	Guinea-caudatum
H40	+/-	-	Fair Red	+	43.87 (like18)	Durra-caudatum

[a] Glumes Color/Pericarp Color, [b] Testa Pigmentation, [c] Seed Color, [d] Panicle Compactness, [e] Grain Weight, [f] Races Classification.

Samples extraction

Of each flour 10 g was defatted twice with hexane at a (4:1) ratio (v/w), and kept on a mechanical shaker for 1h at room temperature. The mixture was centrifuged at 3000 r.p.m for 10 min. The dried defatted flour was then extracted twice with 80% methanol at a (5:1) ratio (v/w) for 1h, at room temperature. After centrifugation at 3000 r.p.m for 10 min, the supernatants were combined. Each extract was subjected to total phenolics, flavonoids and tannin contents determination.

Antimicrobial activity essay was carried out on aliquots of concentrated extracts obtained after evaporating solvent until dryness using a rotary evaporator at 40°C then reconstituted in methanol.

Total phenolics content

Total phenolic content of the sorghum extracts was estimated by the colorimetric assay based on procedures described by Singleton and Rossi (1965) with some modifications [54]. Briefly, an aliquot of the

extract (100 µl) was reacted with 500 µl of 10x diluted FolinCiocalteu reagent and 2 ml of sodium carbonates. The reaction was carried out for 30 min in darkness at room temperature and the absorbance was read at 765 nm. The total phenolic content of each extract was determined from the standard curve and expressed as mg gallic acid equivalent per 100 g of sorghum flour (mg GaE/100g).

Tannin content

The tannin content was estimated using the Vanillin-HCl acid method as described by Price, Van Scoyoc, and Butler (1978) [55] modified by Hagerman (2002) [56]. Briefly, 5 ml of vanillin reagent composed of 0.5% vanillin solution in methanol, acidified with concentrated HCl at 4%, were added to 1 ml of diluted sample. The mixture was allowed to stand for 20 min in 30°C water bath. A set of aliquots of each extract reacting with 4% HCL, was used as blanks to counteract the effect of anthocyanins and other pigments in the samples. The absorption was measured at 500 nm. The absorbance of the blank is subtracted from the absorbance of the corresponding vanillin-containing sample. Tannin content was expressed as mg catechin equivalent per 100 g of sorghum flour (mg CE/100 g).

Total flavonoids content

Total flavonoids content in the extracts was determined by the colorimetric method described by Chang et al. (2000) with some modifications [56]. Briefly, 0.1 g of extract was dissolved in 1 ml of methanol. A 1.5 ml aliquot of this solution was mixed with 1.5 ml of 20 g/l AlCl₃ (6H₂O) and kept at 25°C for 30 min. The absorbance of the reaction mixture was measured at 415 nm. The total flavonoid content of each extract was expressed as mg quercetin equivalent per 100 g of sorghum flour (mg QE/100 g).

Antimicrobial activity

Screening of the antimicrobial activity the crude aqueous methanolic extracts of F11, AS12, AS20, I27 and FZ40 flours was investigated by the disc's diffusion method on an agar medium against two Gram+ bacteria (*Staphylococcus aureus* ATCC 29737 and *Bacillus subtilis* ATCC 6633) bacteria, two Gram-bacteria (*Escherichia coli* ATCC 9739 and *Pseudomonas aeruginosa* ATCC 9027) and one yeast (*Candida albicans* ATCC1023).

Each test strain is inoculated into physiological saline then seeded on the Mueller Hinton agar medium (in the case of bacteria) or Sabouraud medium (in the case of yeast). Sterile 6 mm diameter discs (Whatman N 3 paper) were soaked aseptically with 10 µl/disc of extract prepared in pure methanol at 20 mg/ml. Then, they were deposited on the surface of the agar. The petri dishes are kept at 4°C, for 1 hour in order to allow the diffusion of the extract and momentarily stop the growth of the target germs. After 18 to 24 hours of incubation at 37°C the results were read by measuring the diameter of the light halo around the disc representing the zone of inhibition. Methanol was used as a control.

The minimum inhibitory concentration (MIC) of the five crude extracts was determined by the macro-dilution method in solid media. The method is based on the preparation of a stock solution of each extract, from which dilutions are made at concentrations ranging from 2 to 0.2 mg/ml. For this, each extract is prepared in methanol at a concentration of 20 mg/ml, and then introduced into Muller Hinton medium in order to obtain a stock solution with a concentration of 2 mg/ml. Subsequently, a dilution series of 80, 50, 20 and 10% is carried.

2 µl of suspension of each target seed (previously calibrated at 10⁶ CFU/ml) are deposited spot on the surface of the medium containing a dilution of the extract. The spots are carried out in duplicate and the reading is performed after 24 hours of incubation at 37°C. and consists in evaluating the growth of the bacteria used relative to the controls (medium without extract and inoculated with the same target seeds). The MICs of the products are determined by considering the minimum concentrations that inhibit any growth of germs. The range of target germs was expanded to twenty strains including eight Gram-positive bacteria (*Staphylococcus aureus* ATCC6538, 3 clinical stains of *Staphylococcus aureus* (Sa3, Sa4 and a methicillin resistant *Staphylococcus aureus* MRSA), *Bacillus subtilis* ATCC6333 and *Bacillus cereus* in addition to the actinomycete, *Gardnerella vaginalis*) and five Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC27853, *Pseudomonas fluorescens*, *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* C, and *Enterobacter cloacae*).

Statistical analysis

Measurements of phenolics contents were subjected to a sample-related one-way analysis of variance (ANOVA). For significant F, the post hoc Tukey's range test was used to determine significant differences among means ($\alpha=5\%$).

Results and Discussion

Total polyphenols, flavonoids and tannins contents

The total phenol, flavonoids and tannin levels differed significantly ($P<0.01$) among the sorghum samples. Total polyphenols levels in sorghum extracts ranged between 301.89 ± 0.76 and 3214.46 ± 263.64 mg GaE/100 g of flour, flavonoids levels varied between 4.54 ± 0.85 and 32.03 ± 1.64 mg QE/100 g and tannins levels varied between 6.84 ± 0.01 and 61.54 ± 0.61 mg CE/ g (Table 2).

AS20 extract showed the highest level of both total phenolics and tannin contents but showed similar flavonoids level like I27; while, AS12 presented the lowest values of tannins content and F11 (non-tannin sorghum) gave the lowest values of total polyphenols and flavonoid contents. No significant differences were observed between FZ40 (anon-tannin sorghum) and AS12 total polyphenols and flavonoids contents.

However, no significant differences were observed in flavonoids content between AS20 and I27 samples and between AS12 and FZ40 samples.

Table 2. Total phenolics, flavonoids and tannin contents per 100 g of whole flour of four Algerian sorghum cultivars

Extracts	Total Phenolics (mg GaE/100 g)	Total Flavonoids (mg QE/100 g)	Tannins Contents (mg CE/100 g)
F11	147.62±5.12 ^d	4.54±0.85 ^c	-
AS12	410.03±0.61 ^c	22.38±0.27 ^b	68.4±0.11 ^c
AS20	3214.46±263.64 ^a	32.03±1.64 ^a	615.35±6.10 ^a
I27	1614.57±25.21 ^b	28.22±0.53 ^a	251.14±3.14 ^b
FZ40	301.89±0.76 ^c	20.63±0.43 ^b	-

Values are Means ± δ (n=3). Values within the table followed by different superscript letters are significantly different (p<0.05).

Antimicrobial activity

The aqueous methanolic extracts of *Sorghum bicolor* (L.) Moench whole flours showed a variation in their antimicrobial activity (Table 3). The most important activity is obtained against gram-positive bacteria. The most important inhibition zones were obtained against *B. subtilis* by all the extractions with inhibition diameters that oscillate between 11 and 28 mm. It is interesting to note that the best activity against *B. subtilis* was obtained by the extract AS12 (28 mm) followed by the extract FZ40 (22 mm). Such an important activity was obtained with the F11 extract against *Escherichia coli* ATCC25922 with a diameter of 26 mm. *Candida albicans* was not very sensitive to sorghum extracts with inhibition diameters did not exceed 13 mm. This value was obtained with AS20 and I27 extracts (Table 2).

Table 3. Antimicrobial activity of the crude extracts evaluated by the diffusion method in an agar medium

Target germs	Diameters of the zones of inhibition of the extracts (mm)				
	F11	AS12	AS20	I27	FZ40
<i>Staphylococcus aureus</i> ATCC	13	12	11	13	11

6538

<i>Bacillus subtilis</i> ATCC6333	17	28	15	13	22
<i>Pseudomonas aeruginosa</i> ATCC27853	11	11	12	11	13
<i>Escherichia coli</i> ATCC25922	26	11	11	11	11
<i>Candida albicans</i> ATCC23433	11	11	12	13	11

Determination of the minimum inhibitory concentration of the extracts

The MIC values of the crude extracts vary between 0.2 and 2 mg/ml and sometimes more than 2 mg/ml (Table 4). The lowest MIC (0.2 mg/ml) was obtained against *Streptococcus pneumoniae* (with F11 and FZ40 extracts) and *Escherichia coli* (F11). A MIC of 0.4 mg/ml was obtained against *Staphylococcus aureus* ATCC6538 and methicillin resistant *Staphylococcus aureus* (MRSA) with F11 and AS20 extracts.

All sorghum extracts showed a MIC of 2 mg/ml against *Klebsiella pneumoniae* C, *Enterobacter cloacae* and *Gardnerella vaginalis* strains. However, *Staphylococcus aureus* (Sa3) and *Staphylococcus aureus* (Sa4) could grow on the media containing 2 mg/ml of sorghum extract.

The polyphenols level obtained with the white sorghum, F11, was less important than those reported, by [21], in seven varieties of white sorghum where total polyphenols ranged from 220 to 570 mg GaE/100 g. however, AS20 and I27 polyphenols contents were until four time higher than those reported on other different cereals [22]. Since few years ago, it is recognized that sorghum, millet and barley are important food grains reported to contain significant quantities of phenolic compounds [1, 23, 24].

Table 4. Results of the Minimal inhibition concentration (MIC) expressed in mg of dry extract per ml of agar

Bacteria	F11	AS12	AS20	I27	FZ40
<i>Staphylococcus aureus</i> ATTCC6538	0.4	2	0.4	2	2
<i>Staphylococcus aureus</i> (MRSA)	0.4	1.5	0.4	1	1.5

<i>Staphylococcus aureus</i> (Sa3)	>2	>2	>2	>2	>2
<i>Staphylococcus aureus</i> (Sa4)	>2	>2	>2	>2	>2
<i>Streptococcus pneumoniae</i>	0.2	1.5	0.4	1	0.2
<i>Bacillus cereus</i>	2	2	2	2	2
<i>Bacillus subtilis</i> ATCC6333	1	1.5	1.5	1	0.4
<i>Pseudomonas aeruginosa</i> ATCC27853	1.5	2	1.5	2	1.5
<i>Pseudomonas fluorescens</i>	2	2	2	2	2
<i>Escherichia coli</i> ATCC25922	0.2	2	2	2	2
<i>Klebsiella pneumoniae</i> C	2	2	2	2	2
<i>Enterobacter cloacae</i>	2	2	2	2	2
<i>Gardnerella vaginalis</i>	2	2	2	2	2
<i>Escherichia coli</i> ATCC25922	0.2	2	2	2	2
<i>Klebsiella pneumoniae</i> C	2	2	2	2	2
<i>Enterobacter cloacae</i>	2	2	2	2	2
<i>Gardnerella vaginalis</i>	2	2	2	2	2

Flavonoid contents of the examined cultivars were similar to those obtained by [25] with Egyptian sorghums (45.91-54.68 mg/100g). However, higher levels were, also reported in the literature [26]. Actually, the rate and composition of flavonoids vary with the effect of genotype, environment and their interactions [19, 27, 28].

This variation is function of the pigmentation intensity of both pericarp and testa. Brown sorghums, AS20 and I27, extracts gave higher polyphenols and tannin levelsthan red sorghum, S12 extract. Actually, tannins are the main phenolic compounds in brown tannin sorghums [6].

The high tannin levels in AS20 and I27 indicate that sorghums with pigmented testa have dominant spreader genes (S) while low level of condensed tannins in AS12 indicate that sorghums with a pigmented testa do not have a dominant spreader gene.

The levels of condensed tannins reported in literature, as evaluated by HCL-Vanillin essay, varied from 0 mg to 1.8 mg C.E. per g in tannin type I sorghum but in Type II (with B1_B2_{ss} genes), condensed tannin content varied between 6.4 and 15.5 mg C.E. per g. however, type III sorghums (with dominant B₁B₂S genes) showed much higher levels of condensed tannin ranging from 11 to 50.2 mg C.E. per g [29, 30].

The results of the antimicrobial activity showed more efficiency of aqueous methanolic extracts of sorghums against Gram-positive bacteria than Gram-negative bacteria. Various authors have already reported the susceptibility of Gram-positive bacteria to antibiotics [31, 32] and plant-derived products such as essential oils [33, 34] and organic extracts [35-37]. This is probably due to significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria have an outer membrane and a unique periplasmic space, which is not found in gram-positive bacteria [27]. Concerning *Candida albicans*, a moderate sensitivity was observed to sorghum methanolic extract. In a study on the antimicrobial activity of *Zea mays* (L.), Suket et al. (2014) attributed the effectiveness of the methanolic extract of this plant on the yeast *C. albicans*, to its high content in anthocyanins [38].

The bacteria are classified into 4 categories according to the critical values of the diameters of the zones of inhibition: From 0 to 9 mm: resistant strain; from 10 to 12.5 mm: poorly sensitive strain; from 13 to 20 mm: sensitive strain; More than 21 mm: very sensitive strain [39]. According to this classification, high sensitivity of *Bacillus subtilis* ATCC6333 to AS12 and FZ40 (with DZI of 28 and 22 mm respectively) and *Escherichia coli* ATCC25922 to F11 extract (with DZI of 26 mm) was observed. *Candida albicans* was sensitive to both extracts rich in tannins, AS20 and I27 (with DZI of 12 and 13 mm respectively). *Staphylococcus aureus* ATCC 6538 was sensitive to F11 (13 mm), AS12 (12 mm) and I27 (13 m). *Pseudomonas aeruginosa* ATCC27853 showed sensitivity only to FZ40 extract (13 mm).

Similar MICs were obtained by Gangoué-Piéboji (2009) in their study which included 17 crude extracts of 12 medicinal plants against beta-lactam resistant bacteria [40].

According to the classification of Aligiannis et al. (2001), where antimicrobial activity is considered to be high when MIC values vary between 50 and 500 µg/ml, moderate when they are between 600 and 1500 µg/ml and low when they are above 1600 µg/ml [41]. Thus, all extracts have a moderate to strong activity with a different activity spectrum. Free tannin extracts F11 and FZ40 were the most interesting with MICs between 0.2 and 1.5 mg/l against 7 and 4 test germs respectively (fairly wide spectrum) including Gram-positive and Gram-negative bacteria. However, tannin sorghum extract AS20 and I27 were more effective against Gram-positive bacteria.

However, Okusa et al. (2007) consider extracts with MICs greater than 1000 µg/ml as inactive and only extracts giving MICs less than 500 µg/ml are worth exploring [42]. According to this classification, only 3 extracts (F11, S20 and FZ40) seem to be interesting with an activity directed mainly against *S. aureus* and *S.*

pneumoniae. Nevertheless, it is noted that the F11 extract exerted a strong inhibition against *E. coli* with a MIC of 0.2 mg/ml.

Our results are similar to those obtained by Gordana et al. (2007) concerning the high level of antimicrobial activity exerted by methanolic extracts of sorghum against *S. aureus* strains [43] and to those of Kil et al. (2009) who reported antimicrobial activity and inhibition efficacy of methanolic extract of *S. bicolor* tested on several strains, including *E. coli*, *S. aureus*, *B. subtilis* and *C. albicans* [44].

The variation observed in the antimicrobial activity of methanolic extracts of sorghum can be attributed to the variation of their chemical composition and bioactive compounds such as proanthocyanidins (tannins) and flavonoids and probably of phenolic acids and anthocyanins, in particular, in non-tannin sorghums [45, 46].

The optimal efficiency of an extract may not be due to a main active compound but to the combined action of the different constituents [47]. Actually, FZ40 and AS12 contain same amount of flavonoids but showed different level of antimicrobial activity similar observation was done for AS20 and I27. In addition, a rich tannin sorghum extract gave better results compared to AS12 extracts, containing less tannin but in the same time it was less potent than non-tannin sorghum extracts (F11 and FZ40). Indeed, several authors have reported that the antimicrobial activity is closely related to polyphenol composition [44, 48-50].

The antimicrobial effect of these phenols can be explained by inhibition of bacterial growth following adsorption on cell membranes, interaction with enzymes and effectors or deprivation of substrates and also metal ions [51].

Cowan (1999) has already reported that the different classes of polyphenols, mainly tannins and flavonoids, may increase the toxicity of extracts to microorganisms [13]. This toxicity is a function of the site and the number of hydroxyl groups present on the phenolic compound. In addition, it is obvious that the increase in hydroxylation leads to an increase in toxicity. In a recent study, Bouarab-Chibane et al. (2019) suggest that the antibacterial activity of most of polyphenols depends on interactions between polyphenols and physicochemical properties of bacterial cells surface [52].

Conclusion

These results suggest that antibacterial activity of sorghum extracts is related to the content level versus the composition of phenolics, including tannins and flavonoids compounds. In fact, similar amounts of flavonoids with different amounts of tannins observed in (AS20 and I27) and (FZ40 and AS12) couples were related to different antimicrobial activity spectrum and intensity. We contacted that certain strains were more sensitive than others to in Salah sorghums extracts. They seem to be a potential source of natural anti-streptococcal, anti-staphylococcal and anti-candida substances.

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