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Research Article

ANTIOXIDANT AND ANTIFUNGAL PROFILES OF PHENOL ACID RICH-FRACTIONS FROM *SIDA URENS* L. AGAINST MYCELIA GROWTH INHIBITION OF SOME *ASPERGILLUS* AND *FUSARIUM* SPECIES

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ABSTRACT

Objective: To verify the antifungal capacity of *Sida urens* L.(Malvaceae) traditionally used against mycelia growth inhibition of some *Aspergillus* and *Fusarium* species affecting maize and certain crops or the man by mycotoxins such as aflatoxins responsible of cancers.

Methods: The antioxidant activity of phenol acid-rich fractions has been evaluated using β-carotene–linoleic acid system, 1,1-diphenyl-2-picrylhydrazyl inhibitory activity and chelation of iron (II) ions which showed more pronounced antioxidant capacities of *Sida urens* L. The antifungal effect of phenol acid-rich fractions was studied using Minimum inhibitory concentration (MIC), Minimum fungicidal concentration (MFC) determined by the microdilution and at last the evaluation of Fractional Inhibitory Concentration Index (FICI).

Results: Phenol acid-rich fractions from *Sida urens* L. exhibit maximum radical scavenging activity. As for the Minimum inhibitory concentration assay (MIC) and Minimum fungicidal concentration (MFC) of extract and their combination with standard fungicide (Nystatin), result varied according to microorganism. About FICI, the results indicate a synergistic effect between extract and the standard fungicide (Nystatin).

Conclusions: The results indicated that the phenol acid-rich fraction possessed antioxidant and antifungal properties, and may give credence to some of its ethnopharmacological uses. Therefore, phenol acid-rich fraction of *Sida urens* L., can be effectively employed as antimicrobial agents, specifically antifungal to control growth and prevent colonization and spoilage of food and other plant products with the attendant financial losses by fungi, and prevent the cancers due to the mycotoxins such as aflatoxins.

Keywords: *Sida urens* L., Phenol acid-rich fractions, Antioxidant and antifungal capacity, *Aspergillus* and *Fusarium* species.

INTRODUCTION

The invasion of various food commodities by fungi causes considerable losses in tropical and subtropical countries. *Fusarium* and *Aspergillus* species are common maize pathogens. These species have high specialization on crops, causing sometime stem and ear rot [1]. They are worldwide distributed, mainly in tropical and subtropical areas and have a high survival and a great toxicity [2, 3]. Fungal infections can discolour grain, change its chemical and nutritional characteristics, reduce germination and, most importantly, contaminate it with mycotoxins, such as aflatoxins, and fumonisins which are highly toxic to man and animals [4].

Despite many attempts to control the plants diseases, the problem is still widespread [5]. Phytochemicals are indispensable to fight against plant diseases and to maintain high crop yields. However, indiscriminate use of these chemicals has often resulted in adverse environmental effects, disturbing the ecological balance of soils and making plants even more susceptible to pests and diseases [6]. Increasing public concern on environmental issues requires alternative disease management systems, which are less pesticide dependant or based on naturally occurring compounds [7]. The plants world comprises a rich storehouse of biochemicals to be used as pesticides which are more environmentally safe than synthetic chemicals [8]. There are reports that phytochemicals of *Melia azedarach*, *Euclyptus citriodora* and *Alstonia scholaris* showed fungicidal activity against pathogenic fungi [9]. In a recent study [10] reported that extracts of some rice cultivars exhibit antifungal activity against *Macrophomina phaseolina* and *Ascochyta rabiei*.

According certain studies, it has been established that oxidase stress is among the major causative factors in more than 100 diseases such as malaria, acquired immunodeficiency syndrome, heart disease stroke, arteriosclerosis, diabetes and cancer [11]. In recent years, many studies evidenced that medicinal plants with high content of antioxidants can be effective in prevention of free radical formation by scavenging, thus playing an important role in the prevention of these diseases [12, 13]. The natural active compounds found in medicinal plants belong to various chemical structures including terpenes, alkaloids, coumarins, flavonoids, phenol acids etc. and some of these compounds have anti-cancer, antioxidant and antimicrobial activity [14]. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action because there has been an alarming increases in the incidence of new and re-emerging infectious diseases and resistance to existing antibiotics. About this Malvaceae, no study was carried out on antioxidant and antimicrobial activities of its bioactive fraction (phenol acid-rich fractions) and their antimicrobial alone and in combination with reference antibiotics against *Aspergillus* and *Fusarium* species. As the matter of fact, our aim of the present study was to evaluate the free radicals scavenging capacity and antifungal properties of phenol acid-rich fractions *Sida urens* L. (Malvaceae) and their effects with the reference antibiotics in order to provide a scientific basis for the traditional use of this plant for the management these *Aspergillus* and *Fusarium* species affecting maize and certain crops or the man.

MATERIAL AND METHODS

Plant materials

Sida urens L., was collected in August 2008 in Gampela, 25 Km east of Ouagadougou, capital of Burkina Faso. The plant was botanically identified by Prof. Millogo-Rasolodimby from the plants Biology Department of the University of Ouagadougou. Voucher specimen was deposited in the Herbarium of the La.B.E.V. (Laboratory of Plant

Ecology and Biology, UFR/SVT of University of Ouagadougou) from the University of Ouagadougou.

Fractionation of phenol acids-rich fractions

Fifty grams (50g) of powdered plant material were extracted with 80% aqueous ethanol (500 ml) in 1/10 ratio (w/v) for 24 h under mechanic agitation (SM 25 shaker, Edmund BÜHLER, Germany) at room temperature. After filtration, ethanol was removed under reduced pressure in a rotary evaporator (BÜCHI, Rotavopor R-200, Switzeland) at approximately 40°C. The aqueous extracts were subjected to sequential liquid-liquid extraction with oil ether to remove chlorophyll and other low molecular weight compounds and dichloromethane. This fraction (dichloromethane fraction) was then collected and concentrated to dryness under reduced pressure to obtain phenol acids (dichloromethane fraction). The fraction was freeze-dried by Telstar Cryodos 50 freeze-dryer. The fraction residues were packed in waterproof plastic flasks and stored at 4°C until use. For the tests, lyophilized sample was dissolved with 10% DMSO in water at the desired concentration [15].

Test organisms

The strains of *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Fusarium oxysporum, Fusarium moniliforme* and *Fusarium solani* isolated from maize collected in 2010 in Micorbiology Laboratory of National Institute of Agricultural Research, Ouagadougou/ Burkina Faso following microorganisms all identified by the use of their biochemical profiles as recommended by the manual "Bactériologie Medical" [16] were used as test microorganisms. They were grown on Sabouraud Glucose agar was used for the activation of fungi. Fungal strains were maintained on agar slant at 4°C and sub-cultured on a fresh appropriate agar plates 72 h prior to any antifungal activity. Sabouraud Glucose Agar was used for the activation of fungi. The Mueller Hinton Broth (MHB) was used for the MIC and MFC determinations.

Antioxidant capacity of phenol rich-fractions

β-Carotene–linoleic acid assay

The antioxidant activity of phenol-rich fractions was evaluated using β-carotene–linoleic acid system according to [17]. In short, 1 mL of β-carotene solution in chloroform (0.2 mg/mL) was pipetted into a round-bottom flask. To the solution, 20 mg of linoleic acid and 200 mg of Tween 40 were added. After removing chloroform using a rotary evaporator, 50 ml of aerated distilled water was added to the oily residue. Aliquots (5 ml) of thus obtained emulsion were transferred to a series of tubes containing 2 mg of phenol acid-rich fractions or 0.5 mg of BHA (positive control). Emulsion without antioxidant served as control. After addition of the emulsion to the tubes, they were placed in a water bath at 50 °C for 2 h. During that period, the absorbance of each sample was measured at 470 nm at 15 min intervals, starting immediately after sample preparation (t = 0 min) until the end of the experiment (t = 120 min). The rate of β-carotene bleaching (R) for phenol acid-rich fractions and BHA (standard), was calculated according to first-order kinetics. The percent of antioxidant activity (ANT) was calculated as described in [18], using the equation:

$$\mathbf{ANT = (R_{Control} - R_{Sample})/R_{Control} \times 100}$$

Where $R_{Control}$ and R_{Sample} are average bleaching rates of water control and antioxidant (phenol acid-rich fractions or BHA), respectively.

DPPH radical-scavenging activity

The scavenging effect for DPPH free radical was monitored as described [19] with minor modification. Briefly, 1.0 mL of 0.16 mM DPPH methanolic solution was added to 1.0 mL of either methanolic solution of phenol acid-rich fractions (sample) or methanol (control). The mixtures were vortexed and then left to stand at room temperature in the dark. After 30 min absorbance was read at 517 nm. Radical-scavenging activity (RSA) for DPPH free radical was calculated using the following equation:

$$\mathbf{RSA = (A_{Control} - A_{Sample})/A_{Control} \times 100}$$

Where $A_{Control}$ is the absorbance of the methanol control and A_{Sample} is the absorbance of phenol acid-rich fractions. Synthetic antioxidant, BHA, was used as positive control. DPPH radical-scavenging activity was calculated as the concentration that scavenges 50% of DPPH free radical and thus has RSA = 50% (EC_{50}).

Chelating activity (ChA)

The chelation of iron (II) ions was studied as described by [20]. An aliquot of phenol acid-rich fractions in methanol (1.3 mL) was added to 100 µL of 2 mM FeCl2. After 5 min, the reaction was initiated by adding 200 µL of 5 mM ferrozine. Following 10 min incubation at room temperature, the absorbance at 562 nm was recorded. For preparation of control, 1.3 mL of methanol was used instead of fraction solution. EDTA was used as a chelating standard. The Fe2+ chelating activity (ChA) was calculated using the equation below:

$$\mathbf{ANT = (A_{Control} - A_{Sample})/A_{Control} \times 100}$$

Where $A_{Control}$ is the absorbance of the negative control (solution to which no phenol acid-rich fractions was added) and A_{Sample} is the absorbance of phenol acid-rich fractions solution. Chelating activity was expressed as $ChEC_{50}$, the concentration that chelates 50% of Fe^{2+} ions and thus has ChA = 50%.

***In vitro* antifungal activity**

Preparation of inocula

The fungal strains grown on nutrient agar (Muller Hinton broth) at 35°C for 72 h were suspended in a saline solution (0.9%, w/v) NaCl and adjusted to a turbidity of 0.5 Mac Farland standard **(**$5x10^5$ CFU/ml) [12] .

Preparation of extract substance

The stock solutions of extract were dissolved in 10% dimethylsulfoxide (DMSO) in water [21, 22] at a final concentration of 10000µg/ml. The stock solutions were sterilized by filtration through 0.22 µm sterilizing Millipore express filter.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) was determined by the microdilution method in culture broth as recommended by [21, 23] with low modifications. 12 serial two-fold dilutions of phenol acid-rich fractions solution or conventional antibiotic were prepared as described before, to obtain final concentration ranges of 10000-9.77µg/ml and 50-0.0488µg/ml for phenol acid-rich fractions and standard fungicide respectively. The last wells (n°12) served as sterility controls (contained broth only) or negative control (broth + inoculums). The 96-well micro-plates (NUNC, Danemark) containing 100 µL of Mueller Hinton (MH) broth were used. For each fungi strain, three columns of eleven wells to the micro-plate were used. Each well has getting: the culture medium + phenol acid-rich fractions solution or Nystatin or the combination of phenol acid-rich fractions solution with Nystatin + inoculum standardized at $5x10^5$ CFU/ml (10 µl of inocula) and INT (50 µl; 0.2 mg/ml for 30 min). The plates were sealed with parafilm, then agitated with a plate shaker to mix their contents and incubated at 35°C for 48 h. All tests were performed in triplicate and the fungi activity was expressed as the mean of inhibitions produced. Viable microorganisms reduced the yellow dye to a pink colour. The MIC was defined as the lowest concentration of fraction substance at which no colony was observed after incubation. So, the MIC was defined as the lowest concentration where no change was observed, indicating no growth of microorganism.

Minimum fungicidal concentration (MFC)

Minimum fungicidal concentration (MFC) was determined by the microdilution method in culture broth as recommended by [21, 22] with low modifications. Minimum fungicidal concentration (MFC) was determined by adding 50 µl aliquots of the clear wells to 150 µl of freshly prepared broth medium and incubating at 35°C for 48 h. The MFC was regarded as the lowest concentration of test sample which did not produced a colour. All tests were performed in triplicates.

Evaluation of the Fractional Inhibitory Concentration Index of phenol acid-rich fractions

The Muller Hinton agar dilution method was used to evaluate the Fractional Inhibitory Concentration Index (FICI) of phenol acid-rich fractions and the tested anti-microbial standard as reported earlier [24, 25]. Eleven (11) serial two-fold dilutions of phenol acid-rich fractions solutions were prepared as described before, to obtain final concentration range of 10000 to 9.77 µg/ml. A series of two-fold serial dilutions of Nystatin was also prepared in the same conditions as fraction. In this way, antifungal standard dilutions were mixed with the appropriate concentration of phenol acid-rich fractions solution thus obtaining a series of the combinations of conventional antifungal and phenol acid-rich fractions solution. The concentrations prepared corresponded to 1-1/1024 of MIC values. The 96-well micro-plate (NUNC, Danemark) containing 100µL of Mueller Hinton (MH) broth were used. For each fungal strain, three columns of eleven wells to the micro-plate were used. Each well has getting: the culture medium + combination of phenol acid-rich fractions solution with Nystatin + inoculum standardized at $5x10^5$ CFU/ml (10µl of inocula) and INT (50 µl; 0.2 mg/ml for 30 min). The plates were covered and incubated at 35°C for 48 h. All tests were performed in triplicate and the fungicidal activity was expressed as the mean of inhibitions produced. Viable microorganisms reduced the yellow dye to a pink colour. The analysis of the combination of phenol acid-rich fractions solution and antifungal reference (Nystatin) was obtained by calculating the Fractional Inhibitory Concentration Index (FICI) as follows: FICI= (MICa of phenol acid-rich fractions in combination/MICa alone) + (MICb of the standard antifungal agent in combination/MICb alone), where MICa (Minimal Inhibitory Concentration of phenol acid-rich fractions) and MICb (Minimal Inhibitory Concentration of Nystatin). The FICI was interpreted as follows: (1) a synergistic effect when FICI =0.5; (2) an additive or indifferent effect when FICI > 0.5 and < 1 and (3) an antagonistic effect when >1 [21].

RESULTS

Antioxidant capacity

Antioxidant capacity of β-carotene–linoleic acid assay

The basis of β-carotene–linoleic acid assay is degradation of β-carotene in reaction with linoleic acid free radical. Antioxidants present in the solution can hinder this reaction and consequently prevent discoloration of β-carotene solution. The reduction of absorbance of β-carotene–linoleic acid emulsion was showed in presence of the phenol acd-rich fractions. Comparison of the ANT values of the samples (Figure 1) indicates that the phenol acid-rich fractions were less successful at inhibition of bleaching of β-carotene emulsion comparatively to BHA (P<0.01).

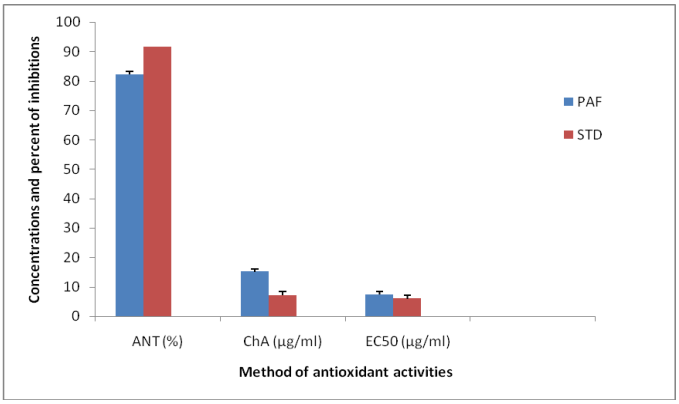

Fig. 1: Antioxidant activity in β-carotene-linoleate test (ANT), DPPH radical scavenging-activity (EC_{50}), and metal chelating activity ($ChEC_{50}$) of phenol acid-rich fractions from *Sida urens* L. and Standards (STD)

Antioxidant of DPPH radical-scavenging activity

The basis of DPPH assay is the discoloration of DPPH• solution in presence of an antioxidant. In its radical form, DPPH absorbs with maximum at 517 nm, but upon reduction with an antioxidant. In this study, phenol acid-rich fractions demonstrated notable antiradical activities albeit lower than the activity of BHA (P<0.05). Results are consigned in the (Figure 1).

Chelating activity (ChA)

The chelating ability of phenol acid-rich fractions toward ferrous ions was investigated (Figure 1) in presence of ferrozin, Fe2+ ion chelator, which upon binding of the metal ionabsorbs with maximum at 562 nm. In the investigated phenol acid-rich fractions demonstrated significant chelating ability in the present research, although lower than the ability of EDTA (P<0.001).

Minimum inhibitory concentration (MIC) assay and Minimum fungicidal concentration (MFC)

As for the Minimum inhibitory concentration assay (MIC) and Minimum fungicidal concentration (MFC) of extract and their combination with standard fungicide (Nystatin), result varied according to microorganism and result is summarise in Table 1, Table 2 and Table 3.

Evaluation of the Fractional Inhibitory Concentration Index of fractions

For FICI, our results indicate a synergistic effect between extract and the standard fungicide (Nystatin) (Table **4**).

Table 1: Minimal Inhibitory Concentration (MIC) of phenol acid-rich fractions from *Sida urens* L., and standard fungicide (Nystatin)

Microorganisms	MIC (µg/ml) extract of *Sida urens* L.	MIC (µg/ml) Nystatin
Aspergillus flavus	78.13±0.00	26.04±0.00
Aspergillus niger	78.13±0.00	26.04±0.00
Aspergillus fumigatus	78.13±0.00	26.04±0.00
Fusarium oxysporum	78.13±0.00	26.04±0.00
Fusarium moniliforme	78.13±0.00	26.04±0.00
Fusarium solani	78.13±0.00	26.04±0.00

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for phenol acid-rich fractions from *Sida urens* L.

Table 2: Minimal Fungicidal Concentration (MFC) of phenol acid-rich fractions from *Sida urens* L., and standard fungicide (Nystatin)

Microorganisms	MFC (µg/ml) extract of *Sida urens* L.	MFC (µg/ml) Nystatin
Aspergillus flavus	104.17±0.00[b]	19.53±0.00
Aspergillus niger	104.17±39.47[b]	19.53±0.00
Aspergillus fumigatus	104.17±39.47[b]	19.53±0.00
Fusarium oxysporum	104.17±0.00[a]	19.53±0.00
Fusarium moniliforme	104.17±0.00[a]	19.53±0.00
Fusarium solani	104.17±33.83[a]	19.53±0.00

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for phenol acid-rich fractions from *Sida urens* L.

Table 3: Minimal Inhibitory Concentration (MIC) of combination of phenol acid-rich fractions from *Sida urens* L., and standard fungicide (Nystatin)

Microorganisms	MIC(µg/ml) Combination of extract + Nystatin
Aspergillus flavus	9.76±0.00
Aspergillus niger	9.76±0.00
Aspergillus fumigatus	9.76±0.00
Fusarium oxysporum	9.76±0.00
Fusarium moniliforme	9.76±0.00
Fusarium solani	9.76±0.00

Values are Mean ±SD (n=3). Different letters in the same column indicate significant difference (P<0.05) for phenol acid-rich fractions from *Sida urens* L.

Table 4: Fractional Inhibitory Concentration (FIC) and FICI of combination of phenol acid-rich fractions from *Sida urens* L.,with Nystatin

Microorganisms	FIC_a	FIC_b	FICI	Effect
Aspergillus flavus	0.12	0.37	0.49	Synergistic
Aspergillus niger	0.12	0.37	0.49	Synergistic
Aspergillus fumigatus	0.12	0.37	0.49	Synergistic
Fusarium oxysporum	0.12	0.37	0.49	Synergistic
Fusarium moniliforme	0.12	0.37	0.49	Synergistic
Fusarium solani	0.12	0.37	0.49	Synergistic

FIC_a = MIC of fraction in combination/MICa alone; **FIC_b** = MIC of the antifungal agent in combination/ MICb alone and FICI= $FIC_a + FIC_b$

a= fraction; b= Nystatin

The FICI was interpreted as follows: (1) a synergistic effect when FICI =0.5; (2) an additive or indifferent effect when FICI > 0.5 and < 1 and (3) an antagonistic effect when > 1.

DISCUSSION

Herbal plants are an important more source of new chemical substance with potential therapeutic uses. Approximately 119 pure chemical substances extracted from higher plants are used in medicine throughout the world [26]. The increase interest on plant medicines in today's world is from belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects [27].

In the present investigation we have studied the antifungal activity of *Sida urens* L. In effect, it is well know that plant products, particularly spices and extracts of various plant parts have been used extensively as natural antimicrobials. [28] reported high levels of inhibition of spore/conidia germination of some fungal species using extracts of rice, wheat straws and tobacco leaf. The above results clearly confirm the fact that phenol acid-rich fractions of *Sida urens* L., has antifungal properties and are able to inhibit the growth of the fungi to different extents. This confirms in vitro activity of some plant extracts. The antifungal activity of certain herbs and plant species against *Aspergillus* sp., has been documented in several research works [29] which was confirmed in the paired mean comparison of plant extracts on growth of all test fungi compared with standard fungicide. The strong inhibition potential of *Sida urens* is attributed to fact that it contains different secondary metabolites [30]. Certain secondary metabolites such as saponins, tannins and flavonoids have been shown to be responsible for therapeutic activity of plants [31]. In effect, according to [32], plant phenols are a major group of compounds acting as primary antioxidants or free radical scavengers due to their hydroxyl groups [33] which contribute directly to the antioxidative action. Phenolic compounds are effective hydrogen donors, making them good antioxidants [34]. Moreover, the antioxidant activity of phenolic compounds is mainly due to their redox properties which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals [35]. Then, our results showed effectively that total phenolics are responsible to the antioxidant capacity and there is a correlation between total phenolics and the antioxidant capacity. Earlier studies have also shown the correlations between polyphenol content and antioxidant [36]. Moreover, plants containing these metabolites usually demonstrate stronger antimicrobial properties than others [37]. Tannins have been reported to inhibit growth of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for them [38]. Saponins are special class of glycosides that have been shown to be an antifungal agent [39]. Plant phenonlic compounds especially flavonoids are currently of growing interest owing to their supposed properties in promoting health [40]. So, certainly, these several chemical constituent increase effectiveness the antimicrobial activity of *Sida urens* L. Additionally, differences in concentrations of extract could account for their antimicrobial potential disparities.

Although the extract mechanism that influences the differences in the susceptibility pattern is not known, it is believed that mycelia cell wall thickness plays a significant role. The adaptation of mycelia for the efficient extraction of nutrients, due to the high surface area to volume ratios is also potentially harmful in absorbing antimicrobial agents and inhibiting growth when present in the medium [41].

Traditionally the plant used for curing various skin diseases and protected harvest against fungi [15]. From the present investigation, the results obtained confirmed the therapeutic potency of *Sida urens* L., used in traditional medicine. The present study also set an important basis for further phytochemical and pharmacological investigation on *Sida urens* L. The present study suggests that the plant extract certainly possess some chemical constituants with antimicrobial properties and this finding is very important in discovering new drugs for the therapy of infectious diseases. However, further studies can be subjected to isolate and characterize the active constituents responsible for the antimicrobial property of *Sida urens* L.

CONCLUSION

The use of *Sida urens* L., could help prevent and preserve the crops and certain plants like maize against fungal contamination and prevent cancers due to the mycotoxins of some *Aspergillus* and *Fusarium* species. Combination of standard fungicide with phenol acid-rich fractions in this study is new antimicrobial method which consisted to evaluate the synergistic effects of plant extract and reference antimicrobials and better fighting against antibiotic resistance.

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