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Phytochemical Screening and Antimicrobial Activities of *Siphonochilus aethiopicus* Extracts from Benin

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Abstract

Siphonochilus aethiopicus (Schweinf.) B.L.Burtt (*Zingiberaceae*), a wild ginger used in traditional medicine in Benin, is investigated for the antibacterial and antifungal activities. Biological activities of ethyl acetate and ethanolic extracts are assessed against six bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*) and three fungi strains (*Aspergillus clavatus*, *Aspergillus ochraceus* and *Aspergillus parasiticus*). Minimal inhibitory concentration is determined by microdilution tests using iodonitrotetrazolium salt as bacterial growth indicator. Inhibition of sporulation and mycelia growth are performed using agar diffusion. *Siphonochilus aethiopicus* is screened for phytochemical constituents. Ethyl acetate extract of the dry rhizome of *S. aethiopicus* showed antibacterial activities against three strains: methicillin resistant *Staphylococcus aureus*, *Staphylococcus aureus* and *Escherichia coli* with a minimal inhibitory concentration of 5 mg/mL. Both extracts are effective on mycelia growth of the three fungi strains with an inhibitory percentage between 40.95±1.65 and 63.50±1.26. The same result is observed for sporulation range from 42.17±0.00 to 61.46±0.006. The phytochemicals alkaloids, flavonoids, triterpenes and steroids are, found in both extracts, and could be responsible of biological activities.

Keywords: *Siphonochilus aethiopicus*, antibacterial, antifungal, *Zingiberaceae*, *Aspergillus*, phytochemical screening.

INTRODUCTION

The Siphonochilus aethiopicus is a specie of *Zingiberaceae*, a large family of flowering plants (Angiosperms) containing about 52 genera for around 1600 species [1] and present in equatorial and subtropical regions [2]. In Africa, they are commonly found in southern tropical areas, particularly in South Africa, Malawi, Zambia [3] and Nigeria [4], where the most common and best known specie is undoubtedly the *Zingiber officinale.* They usually serve as spices or condiments in meals and often used in traditional medicine. In particular, the *Siphonochilus aethiopicus* is known for stimulating women fertility [5] and facilitating erection [6] and is widely used in South Africa by traditional healers, especially by the Zulu. *The Siphonochilus aethiopicus* also has an important role on the inhibition of cyclooxygenase activity in prostaglandin biosynthesis (Cox1 and Cox2) [7]. Previous studies have shown anti-inflammatory role in lungs inflammation, anti-cancer activity against MCF-7 cells [6] and antimicrobial activities on *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* [7, 8]. The essential oils of the roots, rhizomes and leaves of *S. aethiopicus* contain many components such as monoterpenes, sesquiterpenes [9, 4, 10]. Some of these components are active on tumor cell, bacteria, fungi, trypanosome [11].

In Benin, the specie Siphonochilus aethiopicus occurs both in the North and the South and is widely used as therapeutic remedies for various diseases such as hemorrhoids, microbial infections and colds. Due to the importance of the plant and its great use as both food and traditional medicine, it is important to have a better understanding of its therapeutic values. The aim of this study is to investigate particularly the antimicrobial properties of ethyl acetate and ethanolic extracts of the rhizomes of *Siphonochilus aethiopicus* against some selected bacterial and fungal strains responsible for infections and to screen the phytochemical contents.

MATERIAL AND METHODS

Plant Collection

Fresh rhizomes of *Siphonochilus aethiopicus* are collected in Lobogo village, department of Mono in Benin, then identified by a botanist from National Herbarium of Benin (University of Abomey-Calavi). A specimen of the plant had been deposited in the same herbarium and registered under the voucher code AA 6671/HNB. For investigation purpose, the *Siphonochilus aethiopicus* rhizomes are converted into powder using a domestic grinder (Marlex Excella, SEB, France) after the following process: wash, peel, slice and dry at 22°C for two weeks in laboratory.

Preparation of *Siphonochilus aethiopicus* Rhizomes Extracts

185 g of *Siphonochilus aethiopicus* rhizomes powder are extracted with 1 l of ethanol and ethyl acetate. They are macerated on stirring for 72 hours and filtrated using Whatman filter paper N°1, (Qualitative Circles 150 mm, Sigma Aldrich, France). The filtrate is evaporated with Buchi Rotavapor R II (Switzerland). Each extract obtained is weighed into sample bottle and stored at 4°C until the experiments.

Bacterial Strains

Two (2) Gram negative *Eschericha coli* CIP 53126, *Pseudomonas aeruginosa* CIP 82118 and four (4) Gram positive *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* methicillin resistant and *Staphylococcus epidermidis* CIP 8039 are obtained from the Laboratory of Biophotonics and Pharmacology of the University of Strasbourg, France). They are grown in Mueller Hinton Broth (MHB, Merck, Darmstadt, Germany) over night at 37°C on stirring.

Fungal Strains

Aspergillus clavatus, *Aspergillus ochraceus* and *Aspergillus parasiticus* are obtained from the Laboratory of Biochemistry and Molecular Biology at the University of Abomey-Calavi. The fungal spore suspension is prepared in Potato Dextrose Agar (Sigma Aldrich, France).

Phytochemical Screening

Phytochemical screening for major constituents is investigated using the colorimetric method in accordance with the method proposed by Krishnamoorthy and colleagues[5]. Extracts are screened for the presence of alkaloids, anthracenes, anthraquinons, coumarins, flavonoids, saponins, steroids, tannins and triterpenes. Extracts are dissolved in solvents used for extraction except for saponins where water is used. All solvents and reagents used are obtained from Sigma Aldrich, France.

a) Dragendorff reagent is used for alkaloids detection. 1ml of extract at 2.5 mg/ml is mixed with 1 ml HCL 1% and 3 drops of dragendorff. A mix appeared as brown or orange.
b) For anthracene derivatives detection, 1.5 ml of extract at 5 mg/mL is added to 0.5 ml of ammonium hydroxide (NH_4OH) 25% and 0.5 ml of sodium hydroxide (NaOH) 10%. The presence of anthracene derivatives is observed by a red coloration.
c) For coumarins, 1 ml of extract at 10 mg /ml is heated and then cooled. 0.5 ml of ammonium hydroxide (NH_4OH) 25% is added to the cooled extract. The presence of coumarins is shown by a blue-green or purple fluorescence color under UV 254 nm or 366 nm).
d) Test for flavonoids: 1 ml of extract at 5 mg/ml is added to 1 ml sodium hydroxide 25%, (NaOH). A yellow coloration is observed as indication for the presence of flavonoids. It became uncolored when 1 ml of H_2SO_4 10% is added.
e) For saponins, 10 mg of extract is dissolved in 1ml of water. Presence of saponins is indicated by stable persistent froth after vigorous shake.
f) Test for steroids: 5 mg of extract is dissolved in 1 ml of chloroform. Then, 1 ml of acetic anhydride and 1 ml of H_2SO_4 are added. The appearance of a purple red or brownish red ring between the two phases demonstrated the presence of steroids.
g) Test for tannins: 1 ml of extract (5 mg/ml) is added to 3 drops of ferric chloride solution 1%. Tannins are observed when brownish green or a blue-black coloration appeared.
h) Test for triterpenes: the extract is prepared at 5 mg/ml in chloroform. 1 ml of acetic anhydride and 1 ml of H_2SO_4 is added to 1ml of extract. A brownish red color red or purple marked the presence of triterpenes.

Antibacterial Activity

In first step, bacterial sensibility test is realized to eliminate an extract that is inactive at 10 mg/ml according method describing by Eloff in 1998 [12]. Ethanolic and ethyle acetate extracts of *S. aethiopicus* are prepared at 20 mg/ml in acetone and water (v: v). 100 µL of each extract are added in microplates 96 wells with 100 µL of each bacteria inoculum (10^6 UFC/ml) in Mueller Hinton Broth. The microplate is incubated for 18 h at 37°C. 40 µL at 0.2 mg/ml solution of p-iodonitrotetrazolium chloride (INT, Sigma Aldrich Chemie GmbH, Steinheim, Germany) are added in each well. The microplate is incubated during 30 min. The bacterial growth is indicated by the reduction of INT in red color.

Minimum Inhibitory Concentration (MIC)

MIC values of active extracts are determined according Eloff's procedure [13]. The extracts are prepared at 20 mg/ml with a mixture of acetone and water (v: v). 100 µl of Mueller Hinton broth are put in each microplate well, and 100 µl of extract are added to the first line of microplate. Two-fold serial dilutions are realized. The tested concentration ranged from 5 to 0.039 mg/ml. 100 µl of bacteria suspension (10^6 UFC/ml in Mueller Hinton broth) are added to each well. Gentamicin (1mg/ml) is used as standard compound. Two bacterial growth controls are performed (without antibacterial agents and with Acetone/water (v:v)). All tests are realized in triplicate.

The microplates are incubated at 37°C. After 18 h, a solution of 40 µl of p-iodonitrotetrazolium at 0.2 mg/ml (INT, Sigma Aldrich Chemie GmbH, Steinheim, Germany) solution is added and the plates are then incubated again at 37°C for 30 min. MIC is determined as the lowest concentration of plant extract for which the color did not turn red after the addition of INT.

The total activity (TA) values of each extract is determined by dividing the MICs with the quantity extracted from 1 g of the plant material [14].

$$TA = \frac{\text{Qt of extract/1g}}{MIC}$$

TA: Total Activity; Q_t: Quantity of extract from 1 g of plant material; MIC: Minimum Inhibitory Concentration.

Antifungal assay

Three fungi species: *A. parasiticus* CMBB20, *A. ochraceus* CMBB91, *A clavatus*, are used. Each fungus is cultured on Potato Dextrose Agar (PDA, Sigma Aldrich, France). Antifungal activity is evaluated on mycelia development and sporulation stages as described by [15]. Each extract (1mg/mL) prepared in PDA is poured in sterile disposable petri dishes. After solidification, 100 spores prepared in 25% of Tween 20 are deposited on the PDA and the petri dishes are left at 25°C for 5 days. 100 spores are also prepared on PDA without extract as negative control and Fluconazole (100 µg/ml) is used as positive control. Each assay is run in triplicate.

The inhibition percentage (IP) of extracts is determined according to the formula below:

$$IP = \frac{(X-Y)}{X} \times 100$$

IP: inhibition percentage; X: average diameter (mm) of the mycelia or estimated number of spores of control without extract; Y: the average diameter (mm) of the mycelia or estimated number of spores of dishes contained PDA and extract.

RESULTS

Plants Extracts and Phytochemical Constituents

Dry rhizome of *Siphonochilus aethiopicus* is extracted with ethyl acetate and ethanol yielding respectively 3.91% and 5.41 %. The result of phytochemical screening of both extracts is summarized in Table 1. Ethanol or ethyl acetate extracts show the presence of metabolites: alkaloids, flavonoids, triterpenes and steroids.

Table 1: Phytochemical screening of *Siphonochilus aethiopicus* rhizomes.

Secondary metabolite	EtOH	AcOEt
Alkaloids	+	+
Flavonoids	+	+
Triterpenes	+	+
Steroids	+	+
Tannins	-	-
Saponosides	-	-
Coumarins	-	-
Anthraquinones	-	-

(+): Presence of secondary metabolite in the extract
(-): Absence of secondary metabolite in the extract
EtOH: ethanol extract of *Siphonochilus aethiopicus*.
AcOEt: ethyl acetate extract of *Siphonochilus aethiopicus*.

The extractions of *Siphonochilus aethiopicus* from dry rhizomes using ethyl acetate and ethanol resulted in 3.91% and 5.41 % respectively. The summary of the phytochemical screening of both extracts presented in Table 1 highlights the *presence of the same metabolites: alkaloids, flavonoids, triterpenes and steroids*.

Antibacterial Activities

The results of antibacterial tests are given in Tables 2 and 3. Ethyl acetate extract at 10 mg/ml is active against Gram positive bacteria *MRSA* and *S. aureus*. It is also active against Gram negative bacteria *E. coli* with a MIC value of 5 mg/ml for *MRSA* and *S. aureus*. The total activity of this extract is 8 ml for the two strains. Ethanolic extract shows no activity.

Table 2: Effect of ethyl acetate and ethanol extracts of *Siphonochilus aethiopicus* rhizomes at 10 mg/ml against six bacterial strains.

Bacteria	AcOEt	EtOH	Gentamicin
MRSA	+	-	+
Staphylococcus aureus	+	-	+
Staphylococcus epidermidis	-	-	+
Enterococcus faecalis	-	-	+
Pseudomonas aeruginosa	-	-	+
Escherichia coli	+	-	+

(+): active on bacteria; (-): non active on bacteria

MRSA: Methycillin resistance *Staphylococcus aureus*; EtOH: ethanol extract of *Siphonochilus aethiopicus*; AcOEt: ethyl acetate extract of dry *Siphonochilus aethiopicus*

Table 3: Minimal Inhibitory Concentration (MIC in mg/ml) and Total Activity (TA in ml) of dry rhizomes extracts *Siphonochilus aethiopicus*

MIC of extracts	
Bacteria	**AcOEt**
MRSA	5
S. aureus	5
E. coli	>5
Quantity of extract/ 1g	0,040

Total activity of extracts

Bacteria	AcOEt
MRSA	8
S. aureus	8
E. coli	-

EtOH: ethanol extract of dry *Siphonochilus aethiopicus*.
AcOEt: ethyl acetate extract of dry *Siphonochilus aethiopicus*

Antifungal Activities

On mycelial growth and sporulation, the two extracts reveal inhibitory activities, and the most inhibition is obtained for ethyl acetate extract.

Thus, for the mycelial growth, ethyl acetate extract of *S. aethiopicus* is active against *A.clavatus*, *A. ochraceus* and *A. parasiticus* with inhibitory percentage of 41.91%, 51.34%, 63.50% respectively. For the ethanolic extract, inhibition percentage is 40.95%, 41.59% and 57.67% respectively for the same strains. Ethyl acetate extract shows the best inhibition percentage on *A. parasiticus* (63.50%) as presented in Fig. I.

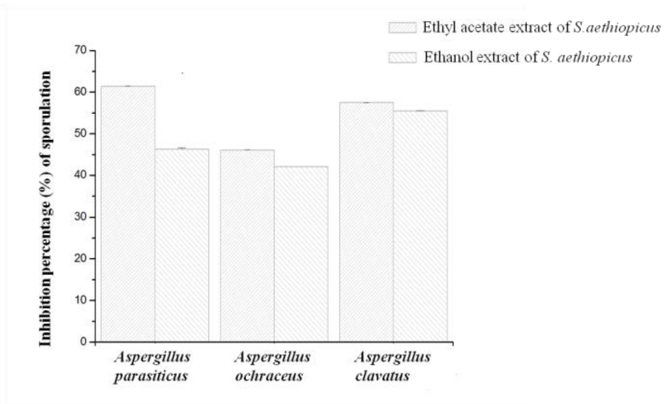

Figure 1: Inhibition percentage (%) of sporulation of three *Aspergillus* strains by *Siphonochilus aethiopicus* extracts (1 mg/ml)

Regarding sporulation, the best activity is observed for ethyl acetate on *A. parasiticus* (57.54%) while the lowest activity is observed for *A. ochraceus* (46.15%). For ethanolic extract, the highest activity is 55.54% on *A. clavatus* (Fig. II). Furthermore, Ethyl acetate also shows better activity on sporulation than observed with ethanolic extract.

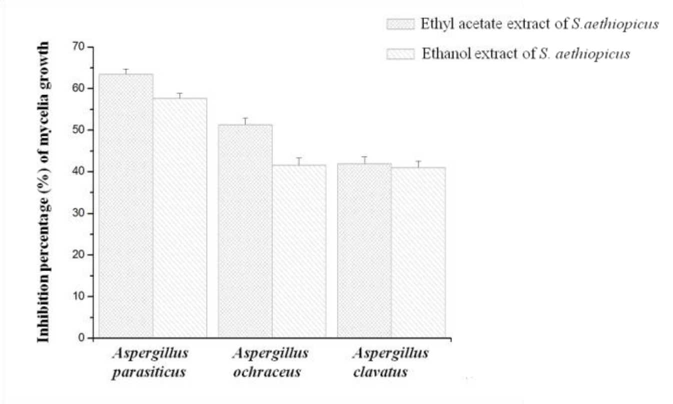

Figure 2: Inhibition percentage (%) of mycelia growth of three *Aspergillus* strains by *Siphonochilus aethiopicus* extracts (1 mg/ml)

DISCUSSION

Phytochemical Activity

Traditional African Medicine uses many plants to treat bacterial and fungal diseases. Many studies have shown that this practice could be explained by the secondary metabolites in these plants [16]. In Benin, *Siphonochilus aethiopicus* is used to manage various diseases but few scientific studies report the activities and phytochemical composition of the extracts of this wild ginger. Our study shows the presence of alkaloids, flavonoids, triterpenes and steroids in the ethanolic and ethyle acetate extracts. To the best of our knowledge, is the first evidence of the presence of alkaloids and flavonoids while a number of terpenoids compounds, such as monoterpenes and sesquiterpenes have already been identified in volatile extracts of fresh rhizomes of *Siphonochilus aethiopicus* in Nigeria, [11] and South Africa [17]. Terpenoids extracts are known for their antitumoral, antimicrobial, antiviral, anti-inflammatory, antiparasitic, and hyperglycemic activities [18]. Alkaloids family represents a large variety of very active compounds known for their antioxidative, antimutagenic, anticarcinogenic and antimicrobial activities [19], while flavonoids are described as antifungal, antibacterial and antioxydant compounds [20].

Although tannins, saponins, anthraquinone and coumarins are not identified in our extracts; Noudogbessi and al (2012) [21] found saponins and tannins in limbo, foliar sheaths and rhizomes of *Siphonochilus aethiopicus* collected in Manigri, North Benin.

Antibacterial Activity

The results of the tested extracts show interesting antibacterial activities. These activities could be justified by the presence of flavonoids and terpenes which are known for their antibacterial properties [22], [23]. Although these compounds are present in both extracts, ethyl acetate extract is more active than ethanolic extract against *MRSA*, *S. aureus* and *E. coli*. This could be due either to other antibacterial compounds present in the ethyl acetate extract or to a molecular interaction between the different compounds in the ethanolic extract that could inhibit the activity of these compounds. It could be also explained by the quantity of the compounds in both extracts.

Several studies report the antimicrobial potential of *S. aethiopicus* [7] and [8]. The ethyl acetate and alcoholic extracts of the various organs, such as leaves, rhizomes and roots of *S. aethiopicus* are found to be active on *E. coli*, *S. aureus*, *B. subtilis* and *K. pneumonia* in South Africa [7]. Our study shows a high MIC value of ethyl acetate extract of rhizome of *S. aethiopicus* (5 mg/ml) as described previously by Coopoosamy and colleagues in 2010 [8]. These results show that ethyl acetate and acetone extracts of rhizomes of *S. aethiopicus* are more active on gram-positive than gram-negative bacteria, with a relative high MIC between 3-4 mg/ml against *Bacillus subtilis*, *Micrococcus kristinae*, *Bacillus cereus* and *S. aereus* [24].

Many antimicrobial activities of other species from *Zingiberaceae* family have been reported. In the study of Srinivasan, wherein 26 species investigated for antibacterial activity, aqueous extracts of bulb, leaves and flowers of *Zingiber officinal* and *Curcuma longa* inhibited bacteria strains, such as: *Chromobacterium, Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Enterobacter faecalis, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella paratyphi* and *S. typhi* [25]. Similarly, the essential oils of leaf and rhizome of *Z. officinale* show antibacterial activity against *Bacillus licheniformis, Bacillus spizizenii, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas stutzeri* [26]. Essential oil of *Curcuma xanthorrhiza* Roxb from Zingiberaceae family showed significant inhibition activity against human pathogenic bacteria: *Klebsiella pneumonia*, *Shigella sonnei* and *Enterobacter aerogens* [27].

Antifungal activity

Ethanolic and ethyl acetate of *S. aethiopicus* show interesting activity against the tested fungi. The antifungal activities could be justified by the verified presence of alkaloids [28] in the extracts. Ethyl acetate extract shows the strongest inhibition of sporulation and mycelia growth of *A. parasiticus, A. clavatus* and *A. ochraceus* fungal strains. Coopoosamy and his colleagues demonstrate that ethanolic extracts of leaves and rhizomes of *S. aethiopicus* inhibit the growth of various fungal strains such as *A. flavus, A. glaucus, C. albicans, C. tropicalis, T. mentagrophytes, T. rubrum*. In same study, the aqueous extracts of rhizomes of *S. aethioipicus* show similar results [8]. Moreover, an antifungal investigation of nine *Zingiberaceae* essential oils, revealed that only rhizome of *Boesenber giapandurata* is active against *Aspergillus niger, Aspergillus fumigatus* and *Mucor* sp. [29]. Essential oils of *Zingiber officinale* exhibit moderate activity against *Aspergillus niger* and *Aspergillus fumigatus* [30].

CONCLUSION

To conclude, this study reports for the first time, the presence of alkaloids and flavonoids in *Siphonochilus aethiopicus* extracts. The presence of terpenoids compounds are also confirmed both in ethanolic and ethyl acetate extracts as in volatile extracts. Ethyl acetate extract of rhizome of *S. aethiopicus* presents better activities on fungi and bacteria compared to ethanolic extract. These results confirmed that the traditional use of *S. aethiopicus* in the treatment of bacterial and fungal infections is indeed effective.

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