

Full Length Research Paper

***In vitro* antifungal and antioxidant activities of two Benin medicinal plants**

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Casuarina equisetifolia (L.) (Casuarinaceae) and *Oxalis corniculata* (L.) (Oxalidaceae), two medicinal plants used in Bénin were screened for their antifungal activity against six strains of the genus *Aspergillus* (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus parasiticus*, *Aspergillus ochraceus* and *Aspergillus nidulans*). The antioxidant activity and phytochemical constituents were also examined. The extracts were screened for the presence of alkaloids, coumarin, anthracene derivatives, flavonoids, lignans, essential oils, naphthoquinones and terpenoids. The antifungal activity was carried out using agar diffusion method, while antioxidant activity was determined by the 2,2-diphenylpicrylhydrazine method. Phytochemical investigation revealed the presence of flavonoids, anthracene derivatives, essential oil, pigments, terpene and triterpene in the leaves of *O. corniculata* and terpene and pigments in the fruits of *C. equisetifolia*. The antifungal activity of extracts is more marked on the sporulation (17.74 to 99.48%) than the mycelia development (7.69 to 65.71%). Methanol and hydro-ethanol extracts showed the best inhibitory percentage of DPPH radical (78 to 95%).

Key words: Medicinal plants, antifungal and antioxidant activity, phytochemical.

INTRODUCTION

Human infections represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide (Kumar et al., 2010). Infections involving the skin and mucosal surfaces constitute a serious problem, especially in tropical and subtropical developing countries (Portillo et al., 2001). Diagnosis and treatment of invasive aspergillosis are one of the unmet needs in medicine today (Lin et al., 2001; Patterson, 2001). Mortality rate can be reduced if the disease is diagnosed and treated at early stage. The treatment of mycoses has lagged behind bacterial chemotherapy and

fewer antifungal than antibacterial substances are available (Duraipandiyar and Ignacimuthu, 2011). Therefore, a search for new antifungal drugs is extremely necessary. The use of herbs in the treatment of man and animal disease has been practiced before the advent of modern antibiotics (Soforowa, 1982). The use of medicinal herbs in parts of the world has also been supported by the isolation of active antifungal compounds from plant extracts (Fabry et al., 1996). Thus, in recent decades, there has been a renewed interest in the use of medicinal plants. Considerable research works have

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been done not only on the pharmacological and phytochemical aspects, but also on the antimicrobial properties of higher plants (Sharma, 2013). Natural products and related structures are essential sources of new pharmaceuticals, because of the immense variety of functionally relevant secondary metabolites of microbial and plant species (Ngo et al., 2013). Approximately half of all drugs that were recorded worldwide in the period before 2007 were from natural products or their synthetic derivatives (Kennedy and Wightman, 2011).

In addition to the diseases caused by microbial infections, people are faced with other diseases including atherosclerosis, arthritis, asthma, cardiac disease, aging, inflammation, and neurodegenerative disorders, which are related to reactive oxygen species (Sini et al., 2010). The reactive oxygen species are used by macrophages to combat infectious agents such as bacteria and viruses. However, the benefits of these highly toxic compounds are not without negative consequence mainly in biological cell structures such as DNA, proteins, lipids (Patel et al., 2010).

Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis (Tsa et al., 2004; Kinghorn et al., 2004). The chemopreventive capacities of plants and natural products with free radical scavenging potential against certain diseases such as ulcers (Borelli and Izzo, 2000), diabetes (Sabu and Kuttan, 2002), memory and cognitive function, Alzheimer's (Howes et al., 2003; Perry et al., 1998), age-related neurological dysfunction (Delanty and Dichter, 2000), cardiovascular and renal disorders (Anderson et al., 1999) have been demonstrated. Indeed, the presence of antioxidant compounds in leaves, bark, roots, fruits and seeds of plant species used in traditional medicine justifies their use in the treatment of these pathologies (Adedapo et al., 2009). Several scientific studies have confirmed the activities of most of the plants used in traditional medicine not only against microbial infections, but also diseases caused by reactive oxygen species (Adedapo et al., 2009; Bolou et al., 2011; Traoré et al., 2012).

In Benin, several plants have been traditionally used for the treatment of a variety of ailments and diseases. Two of these plants, *Casuarina equisetifolia* and *Oxalis corniculata*, are used traditionally to treat skin infections, digestive disorders, dizziness, diarrhea, stomach ache, dysentery, and convulsions. Fruits of *C. equisetifolia* are used alone or in combination with other plants such as *Acacia ataxacanta*, *Vitex doniana*, and *Adansonia digitata*, in the treatment of stomach pain, dental infections, diarrhea, cough, asthma and ulcer. The infusion of the bark and leaves of *O. corniculata* are used in the treatment of intestinal colic, diarrhea, dysentery and other digestive problems. Barcks and fruits of *C. equisetifolia*

and leaves of *O. corniculata* are used by traditional healers for the treatment of mental disorders and as blood purifiers.

The present study aimed to evaluate the *in vitro* antifungal activity of extracts obtained from fruits of *C. equisetifolia* (Casuarinaceae) and leaves of *O. corniculata* (Oxalidaceae) against six species of *Aspergillus*. The antioxidant property was evaluated using the stable free radical diphenylpicrylhydrazine (DPPH). The qualitative phytochemical constituents of extracts were also examined.

MATERIALS AND METHODS

Plant

Fruits of *C. equisetifolia* and leaves of *O. corniculata* were collected by botanist of the National Herbarium of University of Abomey-Calavi in September, 2010 in the area of Ouidah, Department of Atlantic. The collected parts were dried in the laboratory (22±2°C) and reduced to powder using an electric crusher (Marlex electroline Excelled, 3SS GRANDER India 1958).

Extraction

One hundred grams of dry powder of each species were successively extracted by maceration with dichloromethane and methanol for 72 h stirring. A second maceration with ethanol-water mixture (80:20) was carried out with 50 g of dry powder of each species. Each extraction was repeated three times. The macerates were filtered using Whatman No. 1 filter paper. The filtrates were concentrated using a rotary evaporator (RE 300, Stuart) and the extracts were stored at 4°C until biological assay.

Phytochemical screening

Phytochemical screening for major constituents was undertaken using standard qualitative method previously described by Wagner and Bladt (2001). The plant extracts were screened for the presence of alkaloids, tannins, flavonoids, coumarins, lignanes, naphthoquinones, pigments, saponins, anthracene derivatives, terpene, triterpene and essential oil.

Antifungal assay

The following fungi species were used to determine the antifungal activities of extracts: *Aspergillus flavus* CMBB75, *Aspergillus parasiticus* CMBB20, *Aspergillus ochraceus* CMBB91, *Aspergillus nidulans* CMBB90, *Aspergillus terreus* CMBB94 and *Aspergillus fumigatus* CMBB89 obtained from the Laboratory of Biochemistry and Molecular Biology at University of Abomey-Calavi. These are the most common disease-causing fungi of vegetables, animals and humans. The *in vitro* antifungal activity of extracts was screened against mycelia development and sporulation stages of fungi as described previously (Dohou et al., 2004). The tested medians were prepared by pouring the mixture of PDA-extract (1 mg/ml) into sterile petri plates. After solidification, the fungal inoculums estimated at 100 spores were dropping in the petri dishes which were then incubated at 25°C. After 5 days, the diameter of mycelia was measured and the number of spores was counted microscopically. Each assay was performed in triplicate.

Table 1. Phytochemical analysis of fruits of *Casuarina equisetifolia* and leaves of *Oxalis corniculata*.

Chemical constituent	<i>Casuarina equisetifolia</i>	<i>Oxalis corniculata</i>
Alkaloids	---	---
Coumarin	---	---
Anthracene derivatives	---	+++
Flavonoids	---	+
Essential oils	---	+++
Lignans	---	---
Naphtoquinones	---	---
Pigments	+++	++
Saponins	---	---
Terpenes	+++	+++
Triterpenes	---	++
Tannins	---	---

+++ : Presence; ++ : Moderate; + : Traces; --- : Absence.

Three petri dishes without extract were used as control. The inhibitory percentage (IP) of extracts was determined according to the formula that follows:

$$IP = \frac{A_{v \text{ control}} - A_{v \text{ tested dishes}}}{A_{v \text{ control}}} \times 100$$

$A_{v \text{ control}}$ and $A_{v \text{ tested extract}}$ represent the average diameter of the mycelia or estimated number of spores of control and the tested dishes.

Quantitative antioxidant activity

The quantitative antioxidant activity was determined according to the method previously described (Velazquez et al., 2003). Three different concentrations of each extract were tested (100, 10 and 1 µg/ml). Thus, 750 µl of stock solution of each extract and 1500 µl of a 2% DPPH solution in methanol were introduced into dry and sterile tubes. For each concentration, a blank and a negative control were prepared. The blank consists of 750 µl of extract and 1500 µl of methanol. The negative control consists of 1500 µl of the solution of DPPH (2%) and 750 µl of methanol. Each test was done in triplicate and quercetol was used as positive control. The test tubes were incubated in dark at room temperature. After 20 min, the optical density of each mixture was measured at 517 nm using spectrophotometer (Jenway Genova). The inhibitory percentage of DPPH radical which means the antioxidant activity of extracts and quercetol was calculated as follows (Schmeda-Hirschmann et al., 2003).

$$IP = 1 - \frac{A_S - A_B}{A_C} \times 100$$

where IP inhibitory percentage of DPPH; A_S : absorbance of sample; A_B : absorbance of blank; A_C : absorbance of control.

RESULTS

The dichloromethane, methanol and hydroalcoholic

extracts of two medicinal plants, *O. corniculata* and *C. equisetifolia*, were screened for their biological properties and phytochemical constituents.

Phytochemical screening

Phytochemical constituents of leaves of *O. corniculata* and fruits of *C. equisetifolia* are shown in Table 1. The phytochemical analysis of leaves of *O. corniculata* revealed the presence of anthracene derivatives, essential oils, pigments, terpenes, triterpenes and flavonoids, while fruits of *C. equisetifolia* revealed the presence of pigments and terpenoids.

Antifungal assay

The antifungal activity of the extracts was evaluated against mycelial growth and sporulation of six fungal strains of the genus *Aspergillus*. The extracts of the two plants showed significant effect by inhibiting the sporulation of fungal strains. The effect of the extracts at the same concentration is more marked on the inhibition of sporulation than the mycelial growth.

The inhibitory percentage (IP) of extracts on fungi sporulation ranging from 17.74 to 99.48% (Table 2). The methanol extract of *C. equisetifolia* exhibited the strongest activity against *A. fumigatus* with an IP value of 99.48%, while the lowest inhibition was obtained with the dichloromethane extract of *O. corniculata* with an IP value of 17.74% against *A. fumigatus*. Four fungi, including *A. flavus*, *A. ochraceus*, *A. nidulans* and *A. fumigatus* were sensitive to one or more extracts (94% ≥ IP ≥ 99.48%).

Regarding the mycelia development (Table 3), the dichloromethane extract of *C. equisetifolia* has moderate activities against *A. flavus* (53.06%). Similar results were

Table 2. Inhibitory effect of extracts from fruits of *C. equisetifolia* and leaves of *O. corniculata* against *Aspergillus* sporulation.

Extract/Fungi	Inhibitory percentage of sporulation (%)					
	<i>A. flavus</i>	<i>A. parasiticus</i>	<i>A. terreus</i>	<i>A. ochraceus</i>	<i>A. nidulans</i>	<i>A. fumigatus</i>
Ce DM	74.08±0.04	81.93±0.05	84.46±0.02	94±0.03	96.41±0.00	75.49±0.01
Ce Me	63.52±0.04	56.63±0.13	67.67±0.02	79.03±0.02	94.54±0.002	99.48±0.00
Ce H ₂ O/EtOH	62.24±0.09	78.97±0.05	65.55±0.02	92.37±0.03	88.81±0.002	37.46±0.05
Oc DM	37.6±0.19	89.69±0.02	51.78±0.04	63.18±0.17	78.01±0.02	17.74±0.03
Oc Me	94.52±0.23	82.44±0.04	85.8±0.01	92.59±0.03	98.71±0.00	70.14±0.03
Oc H ₂ O/EtOH	92±0.34	82.14±0.06	77.05±0.04	85.11±0.08	94.19±0.00	77.46±0.02

Ce: *Casuarina equisetifolia*; Oc: *Oxalis corniculata*; DM: dichloromethane; Me: methanol; H₂O/EtOH: Water/ethanol; A: *Aspergillus*.

Table 3. Inhibitory effect of extracts from fruits of *C. equisetifolia* and leaves of *O. corniculata* against *Aspergillus* mycelia development.

Extract/Fungi	Inhibitory percentage of mycelia development (%)					
	<i>A. flavus</i>	<i>A. parasiticus</i>	<i>A. terreus</i>	<i>A. ochraceus</i>	<i>A. nidulans</i>	<i>A. fumigatus</i>
Ce DM	53.06±0.01	30±0.00	45.20±0.01	39.65±0.01	10.44±0.06	15.38±0.00
Ce Me	25.51±0.06	30±0.00	43.83±0.04	37.93±0.03	28.35±0.05	23.07±0.00
Ce H ₂ O/EtOH	21.43±0.05	23±0.01	52.05±0.01	50±0.00	52.23±0.01	20.51±0.03
Oc DM	16.32±0.02	48±0.02	52.05±0.02	65.71±0.01	8.95±0.001	7.69±0.00
Oc Me	22.44±0.02	27±0.12	45.20±0.03	37.93±0.18	25.37±0.06	5.12±0.03
Oc H ₂ O/EtOH	47.95±0.16	30±0.00	43.83±0.05	34.48±0.03	19.4±0.02	7.69±0.03

Ce: *Casuarina equisetifolia*; Oc: *Oxalis corniculata*; DM: dichloromethane; Me: methanol; H₂O/EtOH: Water/ethanol; A: *Aspergillus*.

obtained for hydroethanolic extract against *A. terreus* (52.05%), *A. ochraceus* (50%) and *A. nidulans* (52.23%). The dichloromethane extract of *O. corniculata* proved too active against *A. terreus* (IP = 52.05%) and *A. ochraceus* (IP = 65.71%). The other extracts are less active (5.12 to 48%).

Quantitative antioxidant activity

The antioxidant activity of six extracts obtained from fruits of *C. equisetifolia* and leaves of *O. corniculata* was measured for their ability to scavenge DPPH free radicals and results were compared to quercetol which was used as a standard during our test. It was observed that the antioxidant activity of extracts is concentration dependent (Figure 1). At 1 µg/ml, all extracts showed weak activity with IP values ranging from 0.6 to 22.52%, whereas quercetol showed an inhibitory percentage of 74.5%. At 10 µg/ml, four extracts out of six presented an interesting antioxidant activity with IP value ranging from 53.46 to 85%. The methanol extract of *C. equisetifolia* was the most active extract (IP = 85%) compared to quercetol (IP = 75.90%). At 100 µg/ml, five extracts out of six showed interesting activity (75 to 95.68%). The methanol extracts of *C. equisetifolia* and *O. corniculata* were the most active with IP values of 88.97 and 95.68%, respectively, while quercetol showed an IP value of 86%.

DISCUSSION

Medicinal plants are sources of antimicrobial agents, which can be exploited in the management of human diseases. The plants are used medicinally in different countries of the world and are a good source of many potent and powerful drugs (Mahesh and Satish, 2008). Resistance of pathogens to commonly used antifungal agents increased opportunistic infections. This led to increase attention to the search for new therapeutic agents from various sources. Plants are good starting materials for the discovery of new antimicrobial agents (Sasidharan et al., 2011; Saad et al., 2011). In the present study, phytochemical investigation and biological activity of two medicinal plants of Benin Pharmacopeia have been investigated.

The presence of flavonoids, essential oils, terpene and triterpenes in leaves of *O. corniculata* could justify interesting antifungal activity of this species. Flavonoids are the most important groups of secondary metabolites and bioactive compounds present in plants (Kim et al., 2003). They are classified under phenolic groups in plants which have been known to possess antifungal activity (Cowan, 1999). The mechanisms of flavonoids that are antimicrobial can be classified as the inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism (Cushnie and Lamb, 2005). The antifungal effect of the essential oils from several species

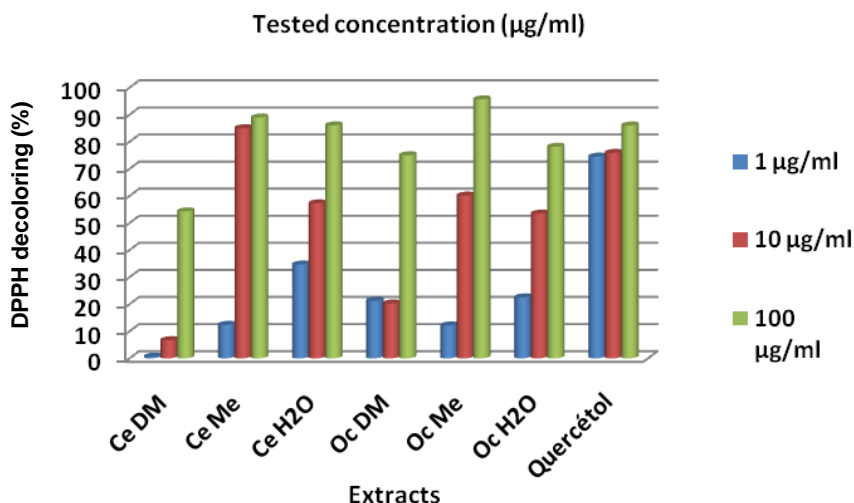


Figure 1. Antioxidant activity of extracts from *C. equisetifolia* (Ce) and *O. corniculata* (Oc). DM: Dichloromethane; MeOH: methanol; H₂O/EtOH: water/ethanol.

has also been demonstrated (Giordani et al., 2004; Romagnoli et al., 2005). The fruits of *C. equisetifolia* also showed antifungal activity against tested fungi. This activity can be attributed to terpenes which were identified during the phytochemical study. Terpenes represent around 55% of the major secondary metabolites of plants (Prakash et al., 2013). A large number of studies have been done in recent years on the antifungal activity of terpenoids of natural origin. A wide range of triterpenes was reported as having antifungal activity (Paduch et al., 2007; Kuete et al., 2007).

Our study showed that the effect of tested extracts at the same concentration is more marked on the inhibition of sporulation than mycelia growth of fungi. Flavonoids have been proven for use against fungal pathogens of man since they have the ability to inhibit spore germination of plant pathogens (Harborne and Williams, 2000; Cushnie and Lamb, 2005). Terpenoids have a role in the regulation of isoprenoid metabolism and signal transduction and as such can exert a profound effect on cell growth, differentiation, apoptosis and multiplication (Harrewijn et al., 2001). It was also found that terpenoids such as farnesyl and geranylgeranyl groups can be built into proteins which play a role in the inhibition of DNA synthesis (Farnsworth et al., 1990; Rilling et al., 1990). Thus, inhibition of sporulation could be due to the presence of flavonoids and terpene in *O. corniculata* and terpene in *C. equisetifolia*.

DPPH is a stable free radical that accepts an electron or hydrogen to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to DPPH and the degree of discoloration indicates the scavenging activity of the drug (Sochor et al., 2010). The decrease in absorbance of DPPH radical caused by antioxidants is due to the

reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation (Thambiraj and Paulsamy, 2012). It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant activity (Jun et al., 2004; Shah et al., 2010).

In this study, most of the extracts showed more than 50% inhibition of DPPH at 100 µg/ml (54.27 to 95.68%). As previously indicated (Aher et al., 2009), our results showed that the anti-radical activity of *C. equisetifolia* is concentration dependent. In recent years, plant extracts have been widely used as natural antioxidants, because of the presence of polyphenolics (Nuengchamrong et al., 2009). Phenolic and flavonoid compounds, contribute to diverse biological activities such as anti-carcinogenic, anti-inflammatory, and anti-atherosclerotic (Khan et al., 2012). In the present investigation, flavonoids and terpenoids have been identified respectively in *C. equisetifolia* and *O. corniculata*. The presence of these compounds could justify their antioxidant activity.

Conclusion

This study confirms the traditional use of *O. corniculata* and *C. equisetifolia* and suggests that some of the extracts contain compounds having antimicrobial and antioxidant properties can be further explored as a possible antimicrobial agent source for the management of infectious pathogenic diseases.

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Conflict of Interests

The author(s) have not declared any conflict of interests

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