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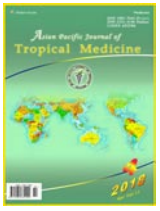

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DPPH antiradical scavenging, anthelmintic and phytochemical studies of *Cissus poulnea* rhizomes

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ABSTRACT

Objective: To investigate the phytochemical constituents, antioxidant and anthelmintic activities of the crude methanol extract of *Cissus populnea* (*C. populnea*) rhizomes. **Methods:** Phytochemical screening was performed using standard protocols, and column chromatography of silica gel was used for the compounds isolation. DPPH antiradical scavenging assay was performed in order to evaluate the antioxidant activity. Total phenolic content was evaluated using the Folin–Ciocalteu assay. The anthelmintic activity was screened on the bovine adult male forms of parasitic nematode *Onchocerca ochengi*, by the *in vitro* evaluation of the inhibition of adult worm motility and mortality. Worms were incubated in the presence of different concentrations of the plant extract and effects on survival were monitored after 24 and 48 h. **Results:** The preliminary phytochemical screening revealed the presence of phenolic compounds, saponins, steroids, tannins, and terpenoids. Bergenin and a mixture of phytosterol, β-sitosterol and stigmasterol were isolated from this extract and were identified by nuclear magnetic resonance, mass spectrometry and by comparison with published data. The crude methanol extract of *C. populnea* rhizomes showed a strong DPPH antiradical activity with a good amount of total phenolic content ((20.69±2.13) g gallic acid equivalent/100 g of extract) and significant anthelmintic activity comparable to the standard drug ivermectin. Bergenin was found to be inactive even after 72 h of incubation. **Conclusions:** This study constitutes the first report on the anthelmintic activity of this plant and supports the traditional use of *C. populnea* as a natural antioxidant and anthelmintic.

1. Introduction

Most of the pathologies known in today world are caused or aggravated by the presence of free radicals in the body. These free radicals generated during metabolism are involved in most of degenerative pathologies such as cancer, diabetes, aging, atherosclerosis, hypertension, and heart attack[1]. It has been reported that subjects with parasitic diseases most often present cases of oxidative stress caused by excessive production of potent reactive oxygen species that can cause tissue damage[2].

Onchocerca volvulus (*O. volvulus*) is a helminth transmitted to humans through the bite of infected *Simulium* blackflies breeding in fast-flowing streams and rivers. The presence of this filarial parasite (death or alive) in the body causes chronic lesions of the

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skin and eyes that can lead to onchocerciasis or river blindness[3]. Onchocercomas are nodule of worms often located in the skin and deeper tissues[3]. Onchocerciasis is still a major cause of blindness in Cameroon. The development of resistance of *O. volvulus* against ivermectin[4] led to a great need to search for new anthelmintic drugs. Several studies reported the efficacy of plant extracts against helminthes[4]. Therefore, anthelmintics from the natural sources may play a key role in the treatment of these parasite infections.

Commonly known as "food gum", *Cissus populnea* (*C. populnea*) is a useful plant playing a very important role in medicine and in nutrition. Belonging to the genus *Cissus* of Vitaceae family, *C. populnea* is one of the most widespread species of the genus *Cissus*[5]. In Africa, the plant is principally found in the Sahelian zones, in the Sudano-Guinean and Guinean savannahs[5]. In Cameroon, it is found in the regions of Adamaoua, North and Far North where it is seasonal. *C. populnea* has always been used as spice[5,6] and as medicinal plant against a broad spectrum of diseases including intestinal parasites, leprosy[5], and malaria[7,8]. The roots of the plant have been reported to be used as anthelminthic[9]. Several pharmacological studies revealed its antimicrobial[9], antiparasitic[7,10], larvicidal[11], hypoglycemic[12] and antioxidant activities[13]. It has been reported that the leaves, stem barks and roots of *C. populnea* contain tannins, flavonoids, saponins, cyanogenic glycosides, alkaloids, anthraquinones, terpenoids and steroids[14-17]. However, there are no reports on phytochemical studies as well as pharmacological studies of the rhizomes of *C. populnea*. The aim of this study was to determine the chemical composition of the crude methanol extract of the rhizomes of *C. populnea* as well as to investigate its *in vitro* antioxidant and anthelmintic activities.

2. Materials and methods

2.1. General experimental procedures

Column chromatography was performed on silica gel 60 (Merck, 70–230 Mesh) with *n*–hexane, ethyl acetate (EtOAc) and methyl alcohol (MeOH) as eluents. The column chromatography was monitored by thin layer chromatography and UV light (254 and 365 nm), H_2SO_4 20% followed by heating at 105 ℃ was used for spots detection. A spectrometer Bruker Avance AV-500 was used for ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) respectively. Tetramethylsilane was used as internal standard, while $CDCl_3$ or DMSO-d_6 was used as solvents. A Q-TOF Ultima spectrometer (Waters) was used for TOF-ESI-MS spectra (ionization voltage 3 kV), while a Rayleigh Vis-723N spectrophotometer was used for absorbance reading.

2.2. Plant material, extraction and isolation

The rhizomes of *C. populnea* Guill & Perr. were collected in September 2014 in Ngaoundere, Adamawa-Cameroon. The plant was authenticated in the Cameroon National Herbarium (CNH), Yaounde (Cameroon) where a voucher specimen (36962/HNC) was deposited. The plant material was dried in the shade and protected from light and moisture in a well-ventilated room at 30 ℃.

A total of 450 g of dried powder was macerated trice in 1.5 L of MeOH with constant shaking 3 times/d for 3 d. After filtration and evaporation of the combined filtrates, 40 g of the resulting crude methanol extract was subjected to column chromatography of silica gel using Hexane/EtOAc (1:0–0:1) and EtOAc-MeOH (1:0–0:1) as solvent to give five fractions (S1 to S5) according to their thin layer chromatography profiles. From the main column, three known compounds, Bergenin (300 mg), and a mixture of β -sitosterol and stigmasterol (15 mg) were obtained. The structures of all the compounds were elucidated by comparison of their spectroscopic nuclear magnetic resonance (NMR) data to those of the literature[18,19].

2.3. Phytochemical screening

The preliminary phytochemical content of extracts was evaluated according to the methods described by Talla *et al*[20] with slight modifications.

2.3.1. Alkaloids

Few drops of Dragendroff's reagent were added to 3 mL of the extract. Red precipitate indicated the presence of alkaloids.

2.3.2. Phenolic compounds

Bluish-black color appearing after adding 2 or 3 drops of a solution of $FeCl_3$ in the dissolved extract indicated the presence of phenols.

2.3.3. Steroids and terpenoids

When an equal volume of acetic anhydride and concentrated H_2SO_4 was added to the extract, the appearance of blue (or green) color or golden yellow color indicated the presence of steroids or triterpenoids respectively.

2.3.4. Saponins

Persistent foam, result of shaking the extract in distilled water for 10–15 min indicated the presence of saponins.

2.3.5. Tannins

For detection, 3–5 drops of a 1% gelatin solution containing NaCl 10% were added to an aliquot of 3 mL of the extract. White precipitates indicated the presence of tannins.

2.4. Determination of total phenolic content

The Folin–Ciocalteu assay was used for the determination of total phenolic content as described by Talla *et al*[20] with slight modifications. Briefly, an aliquot of 0.1 mL of extract (0.2 mg/mL) was allowed to react with 0.2 mL of Folin–Ciocalteu reagent (Sigma, 0.2 mol/L), 2 mL of H_2O for 3–5 min, then 1 mL of 20% Na_2CO_3

(Labosi, 99.5%) and the mixture was incubated at room temperature for 60 min. Absorbances were then taken at 760 nm. Gallic acid was used as standard and results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant material.

2.5. DPPH radical-scavenging assay

DPPH radical scavenging assay was performed according to the protocol described by Jamshed *et al*[21] with some modifications. In a 96-well plate (Costar), 10 μL of sample reacted with 90 μL of DPPH (1.1 mmol/L) then incubated at 37 ℃ for 30 min. After incubation, absorbances were read at 517 nm on a microplate reader. Eight dilutions of each sample were prepared and tested in triplicate. All samples were dissolved in DMSO then dilute with H_2O (final concentration of DMSO <2%), and the DPPH solution was prepared in MeOH. *n*-Propylgallate (Sigma-Aldrich, 97%) and 3-*t*-butyl-4-hydroxyanisole (Sigma-Aldrich, ≥98%) were used as positive control, while 10% DMSO was used as negative control. Percent radical scavenging activity (%) of samples was determined using the formula below:

Percent radical scavenging activity (%) = 100/Absorbance of sample/Absorbance of reference × 100.

2.6. Anthelmintic activity

2.6.1. Extraction and in vitro screening assay of Onchocerca ochengi (O. ochengi) adults worms

Cattle skins containing nodules of *O. ochengi* was carefully washed and sterilized with 10% povidone iodine for dissection[22]. Adult males of *O. ochengi* were then isolated from nodules with collagenase at 37 ℃, washed with phosphate-buffered saline and transferred to the culture medium which consisted of a solution of RPMI-1640 containing penicillin/streptomycin (100 U/100 μg/mL). Six individual worms (one per microliter culture medium in each well) were then incubated with different concentrations of samples in culture medium. A MTT colorimetric assay was used to determine the cell viability of worms, and their mortality was determined after 24 and 48 h. All assays were conducted at 37 ℃ under an atmosphere of 5% CO_2 in humidified air.

2.6.2. Samples preparation and in vitro assay

Stock solutions of samples (4 mg/mL) were prepared in 100% DMSO. In order to determine the lethal concentration of sample required to kill 50% of the worms (LC_{50}), samples were tested in triplicate at six different concentrations obtained by serial dilution starting with a concentration of 1.00 mg/mL. In each well of a 24-well plate, an aliquot of 1 000 μL of each concentration was added to six washed adult worms and then incubated at 37 ℃ in RPMI-1640 medium containing *L*-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL). After 24 and 48 h of incubation, mortality of worms was checked on microscope. Fully elongated and immotile worms were considered as dead. Ivermectin (2.5 mmol/L) prepared in M9-DMSO 10% was used as positive control, while M9-DMSO 10% was used as negative control. LC_{50} values were determined and the results are expressed as mean values±standard error of the mean (SEM).

2.7. Statistical analysis

The results were presented as mean±SEM. Significance between the control and the treated groups were determined by Fisher's *LSD* test and a two-way ANOVA using GraphPad Prism 6 Software, Inc. (San Diego, CA, USA). Significant differences between mean were considered at $P<0.05$.

3. Results

3.1. Phytochemical screening

The result of the phytochemical screening of the crude methanol extract of *C. populnea* rhizomes revealed the presence of phenolic compounds, saponins, steroids, triterpenoids, and tanins except alkaloids.

3.2. Isolation of compounds

Column chromatography of the crude methanol extract of *C. populnea* rhizomes led to the isolation of bergenin, β-sistosterol and stigmasterol. The NMR spectra of these compounds are in accordance with reported data for these compounds.

3.3. Total phenolic content of the extract

The total phenolic content of the extract was determined from the calibration curve of gallic acid ($y = 0.2888\ 8\ x + 0.013\ 4$, $r = 0.993$), and was found to be (20.69±2.13) g GAE/100g of extract (g of Gallic acid per 100 g of extract). This result indicated the presence of a good amount of phenolic compounds in the extract.

3.4. DPPH radical scavenging activity

The crude methanol extract of *C. populnea* rhizomes showed a good DPPH anti-radical activity with an inhibitory concentration 50 (IC_{50}) of 16 μg/mL. However, this activity of the crude extract was significantly lower than that of the standards *n*-propylgallate and *t*-buthylhydroxyanosole [(16.20±0.62) μg/mL *vs*. (5.56±0.22) μg/mL; (16.20±0.62) μg/mL *vs*. (8.84±0.56) μg/mL]. Bergenin with 17.89% of inhibition was considered as inactive.

3.5. Anthelmintic activity

3.5.1. In vitro survival test of O. ochengi in RPMI

This assay was done in order to evaluate the survival ability of the parasites in the culture media RPMI. The survival time of adult male worms of *O. ochengi* was found to be 5 d.

3.5.2. Effect of the crude methanol extract of C. populnea rhizomes on O. ochengi males

The anthelmintic activity against adult *O. ochengi* males was recorded firstly as percent mortality (Figure 1) and then as lethal concentration (LC_{50}). After 24 and 48 h of incubation at 37 ℃ in the presence of different concentrations (0.10, 0.20, 0.40, 0.50, 0.75 and 1.00 mg/mL) of the extract, the mortality rate of worms was found to be time- and concentration-dependent (Figure 1). The extract killed 100% of worms at 1.00 and 0.75 mg/mL respectively after 24 and 48 h. The LC_{50} of the crude methanol extract of *C. populnea* rizhomes against on adult male worms of *O. ochengi* was significantly higher than that of ivermectin ($P<0.05$) respectively after 24 and 48 h [(0.45±0.03) *vs*. (0.30±0.02) mg/mL after 24 h; (0.17±0.02) *vs.* (0.10±0.01) mg/mL after 48 h]. However, no mortality was observed during the experimental period neither in the presence of bergenin nor in the mixture of M9-DMSO, used here as the negative control.

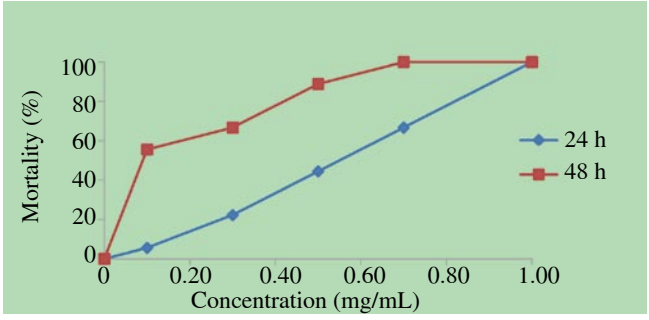

Figure 1. Mortality rates of *O. ochengi* after 24 and 48 h of exposure to the crude methanol extract of *C. populnea* rhizomes.

4. Discussion

The results of phytochemical screening of an extract depend of the solvent used for the extraction. Phenolics, tannins, terpenoids, steroids and saponins revealed in this plant could explain its wide use in traditional medicine, as these families of compounds are well known to possess a broad spectrum of pharmacological activities. Bergenin and the mixture of phytosterol isolated from the crude methanol extract of *C. populnea* rhizomes belong to the family of phenolic compounds and steroids respectively. This confirmed the result of phytochemical screening that revealed the presence of these two families of compounds. Bergenin had already been reported as an anti-diabetic[23], antimalarial[24,25], and cholinesterase inhibitor[26].

The antioxidant activity of bergenin and the crude extract was measured using the DPPH method. The results were expressed by the mean of IC_{50} values for the active samples and by the percentage of inhibition for the inactive ones. After a preliminary screening of all samples, those that showed 50% (or more) of inhibition of the DPPH free radical were diluted and their IC_{50} were calculated. The crude extract was screened at the concentration of 2 mg/mL while bergenin and standards were screened at the concentration of 10 mmol/L. The high DPPH radical scavenging activity of the crude methanol extract of *C. populnea* rhizomes corroborates with the presence in this extract of a good amount of phenolic compounds, and tannins as revealed by the primarily phytochemical studies. The isolated phenolic compound bergenin was found to be inactive against DPPH, and this result corroborates with some previous studies[24,25]. The inactivity of bergenin could be due to the fact that it has only two free –OH phenolic group, with the third one being substituted by –OMe group which is more lipophilic. Moreover, it is known that the more a phenolic compound has adjacent free –OH groups, the more active it is; however, the two –OH groups of bergenin are not adjacent. The thesis of the number of free phenolic –OH groups in bergenin cannot be sufficient to explain its inactivity since one of the positive controls used here (*t*-buthylhydroxyanosole) has only one free –OH phenolic group in its structure. Thus, this observed inactivity of bergenin could therefore also be attributed to the proximity of one of the free phenolic –OH groups of the molecule, to the glucoside group which is a large group and which could thus cause a steric hindrance at the approach of the DPPH radical (which by its structure is also a large group). The antioxidant activity of phenolic compounds depends on the number, the position of hydroxyl groups in the molecule and also on the steric hindrance.

The anthelmintic activity of the extract was determined *in vitro* by the evaluation of the inhibition of adult worm motility and mortality. The results of the present study demonstrated the high anthelmintic activity of the methanol extract of *C. populnea* rhizomes. The anthelmintic activity of the extract against *O. ochengi* may be due to the presence of phenolic compounds and tannins as revealed by the preliminary phytochemical screening of the crude extract, as it has been reported that compounds belonging to these two families of metabolites can cause the death of helminth parasites through a known mechanism[27]. To the best of our knowledge, this is the first study on the anthelmintic activity of *C. populnea* as well as bergenin on *O. ochengi*. The crude methanol extract of rizhomes of *C. populnea* should also be active on the human parasite *O. volvulus*, since *O. ochengi* is biologically the closest species to *O. volvulus*.

The present study assessed the crude methanol extract of the rhizomes *C. populnea* for the *in vitro* DPPH free radical scavenging activity and anthelmintic activity against *O. ochengi*. Our results revealed that the crude extract of the rhizomes of *C. populnea* contains a good level of phenolic compounds, significant free radical scavenging activity against DPPH, and a high *in vitro* anthelmintic activity against the filarial parasite *O. ochengi* which may be due to the presence of this high polyphenolic content. The study also reported for the first time the isolation of bergenin and a mixture of phytosterol from the crude methanol extract of the rhizomes of *C. populnea*. As bergenin, the only phenolic compound isolated in this study, was found to be inactive, further studies may be needed to isolate specific compounds responsible for these antioxidant and anthelmintic properties of *C. populnea* rhizomes. This study therefore supports the traditional use of *C. populnea* as a natural antioxidant and anthelmintic.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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