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A B S T R A C T

Introduction: Drugs resistant *Plasmodium falciparum* is a recurring issue that threatens public health. New anti-plasmodial drugs are needed to overcome this problem. The aim of this study was to characterize *in vitro* an-tiplasmodial activity of *Withania frutescens* (Solanaceae) against chloroquine-resistant *Plasmodium falciparum* strain.

Methods: The *in vitro* antiplasmodial activity of leaves and roots extracts from *Withania frutescens* was performed in 96 well plates and preliminary phytochemical analysis was performed for the active fractions. The toxicity of the plant extract against CHO (Chinese Hamster Ovary) cells was assessed using the tetrazolium salt MTT col-orimetric assay. The selectivity index (SI) was calculated as the ratio between the cytotoxic and antiparasitic activities.

Results: The methanol extract of *W. frutescens* leaves showed a good antiplasmodial activity (CC_{50} 18.1 µg/ml). Furthermore, ethyl acetate and butanol fractions showed promising *in vitro* antiplasmodial activity with a se-lectivity index of 3.1 and 2.4, respectively. The roots of *W. frutescens* were found to be inactive with a CC_{50} value > 80 µg/ml.

Conclusion: The antiplasmodial activity of *W. frutescens* may in part be attributed to the presence of polyphenolic and flavonoid compounds. Based on our results, ethyl acetate and butanol leaves fractions could be considered as a promising source for the development of putative antiplasmodial drugs.

Keywords: Antiplasmodial activity, *Withania frutescens*, *Plasmodium falciparum*, Cytotoxicity, Selectivity index

1. Introduction

Malaria caused by *Plasmodium* parasites is amongst many prevalent public health concerns in several tropical regions of the world. Amongst the five existing species of *Plasmodium* causing malaria in humans, *Plasmodium falciparum* is the most widely studied species since it causes a high number of deaths [1]. In spite of considerable control efforts, malaria remains a major cause of global morbidity and mortality in most tropical countries, especially those in sub-Saharan Africa [2,3]. Resistance to the majority of available antimalarial drugs has been reported in a growing number of countries worldwide and such resistance threatens future progress in malaria control [1]. Medicinal plants have been a reliable source of therapeutic agents and nowadays still represent an inexhaustible pool for the discovery of novel drug leads.

Withania frutescens is a perennial plant mainly distributed in Morocco, Algeria, Spain and Canary Islands [4]. Previous investigation on the pharmacology of this plant showed that its crude extracts displayed an interesting bioactivity profile, possessing various bioactivities including a protective and curative action against carbon tetrachloride (CCl4)-induced hepatotoxicity, neuroprotective properties, antioxidant and cytotoxic effects [5–8]. The major chemical constituents reported from *W. frutescens* are called withanolides. These compounds are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form a δ-lactone. Many of these withanolides are reported to be responsible for the wide array of pharmacological activities, including antitumor, cytotoxic, cancer preventive, antifeedant, anti-inflammatory, leishmanicidal and immune-modulating effects [7,9–13]. In our ongoing research on *W. frutescens*, we aim to explore its antiplasmodial potential in view to discover new drugs. This paper deals, for the first time, with the *in vitro* evaluation of the antiplasmodial activity of *W. frutescens* against a chloroquine-resistant strain of *Plasmodium falciparum*.

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2. Materials and methods

2.1. Plant material

Withania frutescens (L.) Pauquy roots and leaves were collected in El Hassania (20 km from Marrakech), Morocco, in March 2009. The material was authenticated by Prof. A. Abbad, a plant taxonomist, in the Department of Biology, Faculty of Sciences Semlalia, Cadi Ayyad University, Morocco. A voucher specimen (No. mhnm.bk.kh.1) has been deposited at the herbarium of Natural History Museum of Marrakech, Morocco.

2.2. Extracts and fractions preparation

The leaves and roots were separately air-dried in the shade at room temperature (28 °C) until no further changes in their weight was observed (10 days), at Cadi Ayyad University of Marrakech (Morocco), in the laboratory of biotechnology, protection and valorization of plant resources, phytochemistry and pharmacology of aromatic and medicinal unit, and then reduced to fine powder with a grinder. Thus, the powdered parts of plants (100 g each) were exhaustively extracted using a Soxhlet apparatus. The leaves were first defatted with *n*-hexane (400 ml). The roots and defatted leaves were Soxhlet extracted with MeOH for 72 h (400 ml). After removing the solvent under vacuum at 40 °C, the residues obtained from the leaves (25.7 g) and roots (26.3 g) were dissolved in distilled water, filtered and extracted successively with CH_2Cl_2, EtOAc and *n*-BuOH, then concentrated under vacuum to yield 2.7 g, 0.6 g and 3.4 g of the CH_2Cl_2, EtOAc and *n*-BuOH leaf fractions, respectively and 0.9 g, 0.15 g and 3.1 g of the CH_2Cl_2, EtOAc and *n*-BuOH root fractions, respectively.

2.3. Phytochemical analysis of the plant extracts using TLC

The major secondary metabolite classes such as tannins, alkaloids, flavonoids and steroidal lactones were analyzed in *W. frutescens* by thin layer chromatography (TLC), on silica gel 60 F254 Merck. The solvent system used was: hexane/CH_2Cl_2/MeOH (18/3/2, v/v/v) for hexane extract, CH_2Cl_2/MeOH (9/1, v/v) for dichloromethane fraction, EtOAc/MeOH/H_2O (100/20/10, v/v/v) for ethyl acetate fraction and CH_2Cl_2/MeOH/H_2O (65/35/5, v/v/v) for methanol extract and butanol fraction. Visualization of the TLC plates was achieved under UV at 254 and 365 nm and by spraying with NP/PEG reagent for flavonoids, ferric trichloride for tannins and Dragendorff's reagent for alkaloids [14]. The presence of steroidal lactones was revealed with 4-4 nitrobenzylpyridine (NBP) followed by ethylenediamine (EDA) [15].

2.4. Determination of total phenolic compounds

The total phenolic content (TPC) of leaf extracts and fractions was determined using the Folin-Ciocalteu assay [16]. Aliquots of 300 µl containing 100 µg of each sample, dissolved in MeOH, were introduced into test tubes, followed by 1.5 ml of a Folin-Ciocalteu's reagent (diluted 10 times) and 1.2 ml of sodium carbonate (7.5% w/v). The contents of the tubes were mixed and placed in dark for 30 min before the absorbance was measured at 765 nm. The analyses were performed in triplicate and the results were expressed, as *p*-coumaric acid equivalent (CAE), in mg per g of dry extract or fraction ($y = 0.065\ x + 0.057$; $r2 = 0.984$).

2.5. Determination of total flavonoid content

The total flavonoid content of leaf extracts and fractions was determined by the aluminium chloride colorimetric method [17]. In brief, 200 µl of each concentration (5 and 2.5 mg/ml) of the samples in methanol were mixed with 800 µl of distilled water and 60 µl of aqueous solution of $NaNO_2$ (5%). The mixture was allowed to stand for 5 min before the addition of 40 µl of the solution of $AlCl_3$, and the mixture was allowed to stand for 5 min. Then, 400 µl of 1 M of $NaCO_2$ and 500 µl of distilled water were added. The absorbance of all samples was measured at 510 nm. The total flavonoid content was calculated from a calibartion curve, and the result was expressed as mg catechin equivalent per g dry weight.

2.6. In vitro antiplasmodial assay

In this study, *P. falciparum* strain, resistant to chloroquine was used in the *in vitro* culture. Cultures were maintained in fresh A+ human erythrocytes at 2.5% haematocrit in complete medium (RPMI 1640 with 25 mM HEPES, 25 mM $NaHCO_3$, 10% of A+ human serum) at 37 °C under reduced O_2 atmosphere (gas mixture 5% O_2, 5% CO_2, and 90% N_2). Parasitaemia was maintained daily between 1% and 6%.

The *P. falciparum* drug susceptibility test was carried out by comparing quantities of DNA in treated and control cultures of parasite in human erythrocytes according to a SYBR Green I fluorescence-based method using a 96-well fluorescence plate reader. Parasite culture was synchronized at ring stage with 5% sorbitol. Compounds were incubated in a total assay volume of 200 µl (RPMI, 2% haematocrit and 1% parasitaemia) for 72 h in a humidified atmosphere (5% O_2 and 5% CO_2) at 37 °C, in 96-well flat bottom plates.

Triplicate assays were performed for each sample. After incubation, the supernatant was discarded and cells were washed with 150 µl of PBS. 15 µl re-suspended cells were transferred to 96-well flat bottom non sterile black plates (Greiner Bio-one) already containing 15 µl of the SYBR Green lysis buffer (2X SYBR Green, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). Negative control, treated by solvents (DMSO) and positive control (chloroquine) was added to each set of experiments. Plates were incubated for 15 min at 37 °C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission wavelengths at 497 and 520 nm, respectively. The concentrations of compounds required to induce a 50% decrease of parasite growth (CC_{50}) were calculated from three independent experiments.

2.7. In vitro cytotoxicity evaluation on CHO cell line

The evaluation of the tested extracts cytotoxicity was conducted on the Chinese Hamster Ovary (CHO) cells according to the method described by Mosmann [18] with slight modifications. Briefly, cells in 100 µl of complete medium, [RPMI supplemented with 10% foetal bovine serum, 1% L-glutamine (200 mM) and 1% penicillin (100 U/ml)/streptomycin (100 µg/ml)], were inoculated into each well of 96-well plates and incubated at 37 °C in a humidified 6% CO_2, 14% O_2 and 80% N_2 atmosphere. After 24 h incubation, 100 µl of medium with various product concentrations was added and the plates were incubated for 72 h. At the end of the treatment and incubation, each plate-well was microscope-examined for detecting possible precipitate formation before the medium was aspirated from the wells. 10 µl of MTT solution (5 mg MTT/ml in PBS) were then added to each well with 100 µl of medium without foetal calf serum. Cells were incubated for 2 h at 37 °C to allow MTT oxidation by mitochondrial dehydrogenase in the viable cells. After this time, the MTT solution was removed and DMSO (100 µl) was added to dissolve the resulting blue formazan crystals. Plates were shaken vigorously (300 rpm) for 5 min. The absorbance was measured at 570 nm with a microplate spectrophotometer (ELX 808, IU, BIOTEK). DMSO was used as blank and doxorubicine as positive control. Cell viability was calculated as percentage of control (cells incubated without compound). The 50% cytotoxic concentration was determined from the dose–response curve.

2.8. Selectivity index (SI)

The selectivity index (SI), which corresponds to the ratio between

Table 1
In vitro antiplasmodial and cytotoxic activities of *Withania frutescens* methanol extracts on W2 (CQ-R) strains of *Plasmodium falciparum.*

	CC_{50} CHO (µg/ml)	CC_{50} Plasmodial (µg/ml)	SI
Leaves	14	18.1	0.8
Roots	> 100	86.8	> 1.2
Chloroquine	–	0.13	–
Doxorubicine	0.98	–	–

Chloroquine was used as positive control for antiplasmodial activity and Doxorubicine was used as positive control for cytotoxicity activity.

the cytotoxic and antiparasitic activities of each tested samples, was calculated to assess the selectivity of samples to *P. falciparum.*

3. Results

The antiplasmodial activity of the methanol extracts obtained from *W. frutescens* leaves and roots was tested against chloroquine resistant (W2) strains of *Plasmodium falciparum*. The *in vitro* antiplasmodial assay revealed that the methanol leaves extract exhibited an interesting activity against the W2-R *Plasmodium falciparum* strain with a CC_{50} of 18.1 µg/ml (Table 1). Whereas, the methanol roots extract was shown to be inactive with a $CC_{50} > 80$ µg/ml.

To further characterize the chemical class associated with the observed antiplasmodial activity, different organic fractions we prepared from the methanol leaves extract using solvents with different polarity. Each fraction was then tested against W2-R *Plasmodium falciparum* strains.

The most potent antiplasmodial activity was observed in the CH_2Cl_2 (CC_{50} 9.4 µg/ml) followed by butanol (CC_{50} 12.4 µg/ml) and ethyl acetate (CC_{50} 22.3 µg/ml) fractions. However, the selectivity indexes were proportionally inversed to the determined inhibitory concentration (Table 2).

The result of the phytochemical screening showed the presence of flavonoids in ethyl acetate and butanol fractions, steroidal lactones were detected in dichloromethane and butanol fractions. Based on the polarity of the used solvents, dichloromethane and butanol fractions probably contain free withanolides and glycowithanolides, respectively [19], while none of the tested extracts and fractions contained tannins or alkaloids.

Phenolic compounds including flavonoid are one of the most important classes of antiplasmodial compounds [20]. The ethyl acetate fraction possess high phenolic and flavonoid content (254.8 ± 2.9 mg CAE/g dry fraction and 122 ± 0.77 mg CE/g dry fraction, respectively) which may be responsible for its antiplasmodial activity. The concentration of flavonoid in butanol fraction (186.7 ± 0.12 mg CE/g dry fraction) was more significant. Therefore, these compounds might have improved the antiplasmodial activity of this fraction (Table 3).

Table 2
In vitro antiplasmodial activity of different fractions of methanol leaves extract on W2 (CQ-R) strains of *Plasmodium falciparum.*

		CC_{50} CHO (µg/ml)	CC_{50} Plasmodial (µg/ml)	SI
Leaves	CH_2Cl_2 F.	7	9.4	0.7
	EtOAc F.	70	22.3	3.1
	BuOH F.	30	12.4	2.4
Chloroquine		–	0.13	–
Doxorubicine		0.98	–	–

CH_2Cl_2 F.: dichloromethane fraction; EtOAc F.: ethyl acetate fraction; BuOH F.: *n*-butanol fraction.
Chloroquine was used as positive control for antiplasmodial activity and Doxorubicine was used as positive control for cytotoxicity activity.

Table 3
Total phenolic content (TPC) and total flavonoid content (TFC) of leaves extracts and fractions of *W. frutescens.*

Samples	TPC (mg CAE/g dry extract or fraction)	TFC (mg CE/g dry extract or fraction)
Hexane E.	26.61 ± 1.4e	9.87 ± 0.43e
MeOH E.	144.15 ± 3.73c	133 ± 0.13b
CH_2Cl_2 F.	32 ± 1.07d	19 ± 0.3d
EtOAc F.	254.77 ± 2.88a	122 ± 0.77c
BuOH F.	200.61 ± 0.96b	186.7 ± 0.12a

Hexane E.: hexane extract; MeOH E.: methanol extract; CH2Cl2F.: dichloromethane fraction; EtOAc F.: ethyl acetate fraction; BuOH F.: butanol fraction. ACE: *p*-coumaric acid equivalent; CE: catechin equivalent. All assays were determined in triplicates ± standard deviation. For each assay, values followed by the same letter are not significantly different at $P < 0.05$, as measured by the SNK test.

4. Discussion

According to Jonville et al. [21] antiplasmodial activity has been classified as follows: high ($CC_{50} < 5$ µg/ml), promising ($5 < CC_{50} < 15$ µg/ml), moderate ($15 < CC_{50} < 50$ µg/ml) and inactive ($CC_{50} > 50$ µg/ml). This is the first report showing antiplasmodial activity of *W. frutescens*. From the obtained data, it appears that methanol extract of *W. frutescens* leaves exhibited good activity *in vitro* against chloroquine resistant strains (W2-R) of *Plasmodium falciparum*, whereas methanol roots extract was inactive (Table 1).

The obtained fractions from leaves methanol extracts of *W. frutescens* displayed promising activity on the tested strain (CC_{50} 9.4–22.3 µg/ml). The dichloromethane fraction exhibited the highest antiplasmodial activity with a CC_{50} of 9.4 µg/ml, nonetheless it exhibited significant cytotoxic activity against CHO cell lines. Our previous study has shown that the dichloromethane fraction of *W. frutescens* leaves exhibited cytotoxic activity against cancer cell lines (Hep G2), which can be ascribed to the phytochemical constituents; the withanolides [7]. Earlier studies reported the antiplasmodial activity of some other sesquiterpenoid lactones, but in both reports high cytotoxicity was also associated with the compounds [22,23].

The ethyl acetate and butanol fractions enriched in polyphenols and flavonoids proved to be active against malaria with a promising to moderate activity (specificity index of 3.1 and 2.4, respectively). Numerous studies have been reported wide varieties of pharmacological activities of polyphenols and flavonoids, including antiplasmodial activity [24,25]. Antiplasmodial activity has been reported for some biflavonoids primarily (I-30, II-8)-biflavones, in which a high number of methoxy substituents were associated with high activity [26]. This group enhances the lipophilicity of the substance, improving its incorporation into cells [27]. It is concluded from of the present study that the ethyl acetate and butanol fractions of *W. frutescens* leaves possess a promising antiplasmodial activity. This activity may be ascribable to high level of polyphenols and flavonoids in the fractions. Pure drugs that are industrially produced or isolated from plants against a humans disease have not always the same degree of activity as the unrefined extract or fraction at comparable concentration of the active component, and in many cases there is evidence of synergistic or multifactorial effects between compounds present in the extracts [28,29]. In a recent study, it was found that the antiplasmodial IC_{50} values of *Artemisia annua* extracts were 6 to 18 fold lower than was expected in terms of their artemisinin content, suggesting that the activity of the extracts could not be accounted entirely by their artemisinin content [29]. In another study, the flavone casticin enhanced the *in vitro* activity of artemisinin by 3–5 fold [30]. This result demonstrates that the mode of action of artemisinin depends on the formation of free radicals as a consequence of the interaction of haem iron in malaria infected red bood cells with the endoperoxide moiety of artemisinin [31]. Thus, alternative antiplasmodials must be needed to overcome any deficiencies in present therapy. In this context, further study should be conducted for the purification of bioactive fractions of *Withania frutesence* in regard to isolate active antiplasmodial compounds and thereafter the investigation of the synergistic interaction between the isolated compounds and also between them and the commercial antimalarial drugs.

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