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Full Length Research Paper

Incidence of polypharmacy in Alzheimer's disease elderly patients from Guarapuava City (Paraná, Brazil)

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Alzheimer's disease (AD) affects a large portion of the elderly worldwide and is the most common dementia in this population. AD is usually accompanied by concurrent comorbidities leading to the simultaneous use of several drugs to improve the quality of life, which renders AD patients vulnerable to drug interactions and adverse reactions. This study assessed the frequency of polypharmacy based on comorbidities in AD patients from the city of Guarapuava, Paraná, Brazil. This is a cross-sectional study in non-institutionalized and volunteer AD patients. The Clinical Dementia Rating (CDR) scale was applied to classify the AD stage and a socio-economic survey was used to identify possible comorbidities and medications taken. Medications were evaluated according to the Kussano's criteria (2010) to identify polypharmacy. The incidence of polypharmacy was high (up to 65.9%, n = 27); hypertension was the most frequent comorbidity (58.54%, n = 24). AD patients inappropriately take drugs, either because of inattention or lack of popular medical understanding and may be subjected to consequences such as drug interactions and iatrogenic adverse reactions. Hence, studies focusing on AD patients investigating further risks caused by drug interactions are relevant and can increase awareness in their health care assistance and caregivers.

Key words: Alzheimer's disease, polypharmacy, iatrogeny, adverse reactions.

INTRODUCTION

The number of people over 65 years old has grown significantly in the last decades in Brazil (Silva et al., 2012; Secoli, 2010). According to the Brazilian Institute of Geography and Statistics (IBGE), the population in this age group should increase from 14.9 million (7.4% of the total population) in 2013 to 58.4 million (26.7% of the total

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population) in 2060, with a life expectancy of 81 years. This estimate represents four times the current elderly population in Brazil. Other figures show that about one in five Americans will be over 65 years old in 2030, which leads to the estimation that 88.5 million Americans will be 65 years and older in 2050 (Oster and Oster, 2015). This increased life expectancy has resulted from the emergence of new primary prevention programs for diseases and advances in medical technology (Silva et al., 2013).

An increase in the occurrence of diseases associated with senility occurs with an increase in life expectancy. Thus, Alzheimer's disease (AD) appears as the most common dementia affecting more than 20 million elderly worldwide (Pinheiro et al., 2013). Dementias are often accompanied by comorbidities such as diabetes, hypertension, congestive heart failure and deglutition disorders among others (Caixeta et al., 2012) and can affect about 40 to 56% of people with cognitive decline (Martín-Garcia et al., 2013).

Due to the pathological process of dementia and its comorbidities, the concurrent use of daily multiple medications becomes common practice to improve the quality of life in this group (Silva et al., 2012; Kusano, 2009; Pinheiro et al., 2013; Quinalha and Correr, 2010; Secoli, 2010). This practice makes the elderly more vulnerable to adverse effects that can result from taking multiple drugs (Kusano, 2009; Ribeiro et al., 2014).

Concomitant use of multiple drugs is called polypharmacy and it can be classified as mild, moderate or severe according to the number of drugs used by the patient. The mild condition is defined as the use of two or three drugs, the moderate condition as the use of four to five drugs, and the severe as the use of more than five drugs (Silva et al., 2012; Kusano, 2009). Some studies have demonstrated that the prescription of more than two drugs can provoke adverse reactions due to drug interactions between active ingredients or formula components such as excipients and flavoring agents (Colette et al. 2011; Tavares et al., 2013).

Based on the possibility of the occurrence of polypharmacy and the lack of studies evaluating the frequency in the use of multiple concomitant medications, this study assessed the frequency of polypharmacy according to the occurrence of comorbidities in AD patients who are residents of the city of Guarapuava, Paraná, Brazil.

MATERIALS AND METHODS

The study followed the quantitative order of the cross-sectional type. The sample consisted of elderlies assisted at the Unified Health System (SUS) in the city of Guarapuava, Paraná, Brazil, who were identified through the computerized system of the Information Technology and Communication Company of Paraná (CELEPAR®). The study was approved by the Ethics Committee on Research involving humans from the Midwest State University under the opinion number of 6111316/2014.

After data collection in the system, the elderlies were contacted by phone to arrange for home visits between January and October 2014. The Voluntary Informed Consent Form - TCLE – was delivered and signed by the corresponding patients' guardians or caregivers. The final disease diagnosis was made through a histological examination of postmortem brain tissue. Thus, the American Psychiatric Association (DSM) and the "National Institute for Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association" (NINCDS-ADRDA) recommend that the life diagnosis of dementia should be performed through a proper research-based history and general physical examination. In this study, the most cited neurological examination, the "Clinical Dementia Rating" (CDR) (Oliveira et al., 2008) was used to track the disease stage. This rating is divided into CDR 1, CDR 2, and CDR3, which indicate the dementia severity as mild, moderate and severe, respectively.

The sociodemographic questionnaire, which was structured to draw the study population's profile, was also applied to identify existing comorbidities, and drugs, dosage and frequency of usage. All the drugs, whether prescribed or self-medicated, were included in the study. Polypharmacy was classified according to Kusano (2009) as mild (use of two or three drugs), moderate (use of four or five drugs) and severe (use of more than five drugs).

The sample inclusion criteria were the presence of AD diagnosed by a geriatrician or neurologist, and participation in the "Specialized Component of Pharmaceutical Assistance" program (CEAF) provided by the 5th Regional Health of Guarapuava, Paraná. The exclusion criteria were patient not found after three home visits and death before study completion.

Data were analyzed using the SPSS version 20.0 statistical package for Windows®. The results were presented in relative and absolute frequencies. The Chi-square test, Fisher's exact test, and Pearson correlation coefficient were used to investigate possible associations. The significance level of $P < 0.05$ was adopted.

RESULTS

Out of the 57 AD patients initially selected, 8 (14.04%) were not found, and 8 (14.04%) died before the study completion; results were obtained for 41 (71.93%) patients. The sociodemographic data from these patients are presented in Table 1.

Polypharmacy was identified in 65.9% (n = 27) of the patients, mostly occurred in groups CDR 2 and 3 (Table 2) and resulted from simultaneous administration of drugs due to the presence of comorbidities. The Pearson linear correlation coefficient indicates a moderate correlation between polypharmacy incidence and lack of incidence.

The Pearson linear correlation coefficient indicates a moderate correlation between polypharmacy incidence and lack of incidence. Table 3 shows the analysis of pharmacological classes of the drugs used by the elderlies.

One of the pharmacological strategies in the treatment of AD is making use of acetylcholinesterase enzyme inhibitors when two drugs are the main treatment protagonists; in this study (Table 4), more than half of the elderlies (54.84%, n = 17) used the drug Donepezil hydrochloride.

According to the drugs used for self-declared patho-

Table 1. Sample design.

Gender	Percentage	Mean age	CDR		
			1	2	3
Male	39.02% (n=16)	79.27 ± 8.20	18.75% (n=3)	37.5% (n=6)	43.75% (n=7)
Female	60.98% (n=25)	77.70 ± 14.12	12% (n=3)	40% (n=10)	48% (n=12)
		Total	14.64% (n=6)	39.02% (n=16)	46.34% (n=19)

Data presented as mean ± standard deviation; relative frequencies.

Table 2. Correlation between polypharmacy and the CDR scale.

Polypharmacy	CDR 1	CDR 2	CDR 3	*P*
	Mild	Moderate	Severe	
No	4.9% (n=2)	9.8% (n=4)	19.5% (n=8)	0.678
Yes	9.8% (n=4)	29.3% (n=12)	26.8% (n=11)	

Data presented as relative frequencies; Pearson linear correlation coefficient through the Chi-square and Fisher's exact tests.

logies, hypertension was the most frequent comorbidity in these elderlies, with 24 (58.54%) cases (Table 5). The comorbidities with the lowest incidences were Parkinson's disease and stroke, with 7 (17.7%) cases with each disease.

DISCUSSION

The current study findings corroborate with those reported by Lucchetti et al. (2010) and Hanlon et al. (2009). Lucchetti studied a sample of 209 patients in a Brazilian hospital and verified the occurrence of polypharmacy in 46.4% (n = 97). In this same study, 67.9% of the patients (n = 142) were affected by hypertension and used cardiovascular, psychotropic, anticonvulsant and antidepressant drugs. Hanlon et al. (2009) observed the occurrence of 74% polypharmacy when studying 113 care institutions in the United States. Therefore, it is evident that the elderly are, in general, potentially susceptible to the risk of adverse effects caused by drug interactions and idiosyncratic reactions (Nguyen, 2006).

Carvalho et al. (2012) observed that the high prevalence of polypharmacy in elderlies was due to the presence of chronic non-transmissible diseases (NTDs) when studying 2,143 elderlies and observed that 36% of the group took more than five drugs, which is characterized as severe polypharmacy. This result is consistent with our study; elderlies suffer from multiple medical problems, including AD, which affects the quality of life. So, polypharmacy could be expected in the elderly population at large, not only elderly AD patients. Polypharmacy could be more dangerous to AD patients because of their dementia. According to Rozenfeld et al. (2008), diseases such as hypertension, heart diseases, rheumatic diseases and diabetes indicate the potential for polypharmacy because patients with these diseases need several medications with proven effectiveness in order to achieve health improvement. This assumption explains the high number of cases found in this study.

By comparing the drugs used in this study with the criteria of Beers et al. (1991), it was detected that some drugs, such as benzodiazepines (Alprazolam and Diazepam), antidepressants (Fluoxetine), antihistamines (Cyproheptadine) and some antihypertensive (Doxazosin) are used inappropriately, imposing a high degree of side effects and drug interactions. Such substances could be replaced by others that are appropriate for the treatment and do not cause potential side effects. Furthermore, according to the classification of Secoli (2010), some interactions among the most common classes of drugs were identified in this study such as between the antihypertensive captopril and the diuretic hydrochlorothiazide, and between the antiarrhythmic amiodarone and the anticoagulant Warfarin; the potentiation of these drugs by the concomitant use of captopril leads to inhibition of cytochrome P450.

Hypertension is the most common comorbidity in the elderly population as shown in Table 5. According to Regalado Doña et al. (2009), this constitutes a risk factor for those with vascular disease and AD. In this study, more than half of the elderlies suffered from hypertension and required blood pressure treatment with drugs. The smallest possible number of drugs should be used considering the iatrogenic/benefits risk ratio. Based on the risks, caution in choosing the anticholinesterasic drug

Table 3. Pharmacological classes most frequently used by AD patients in the city of Guarapuava, PR.

Class	Number of individuals	%
Antihypertensive	32	78.05
Anticholinesterasic	30	73.17
Antiparkinsonian	21	51.22
Antidepressant	18	43.90
Antidiabetic	18	43.90
Analgesic	17	41.46
Antipsychotic	16	39.02
Antihyperlipidemic	12	29.27
Supplement	10	24.39
Antiulcer	9	21.95
Diuretic	9	21.95
Anticonvulsant	6	14.63
Antiplatelet	5	12.20
Vasodilator	5	12.20
Anxiolytic	5	12.20
Thyroid hormone	5	12.20
Antiarrhythmic	4	9.76
Antithrombotic	4	9.76
Alpha-blocker	3	7.32
Anti-alopecia	2	4.88
Others	16	39.04

Data presented as relative frequencies.

Table 4. Anticholinesterasics used by the studied elderlies.

Drug	Number of individuals	%
Donepezil hydrochloride	17	54.84
Rivastigmine hemitartrate	13	41.94

Data presented as relative frequencies.

Table 5. Observed comorbidities.

	Number of individuals	%
Hypertension	24	58.54
Diabetes	12	28.27
Hypercholesterolemia	11	26.83
Cancer	8	19.51
Parkinson's disease	7	17.7
Cerebrovascular accident	7	17.7

Data presented as relative frequencies.

is necessary to avoid the prescription of those drugs in this class that has a central action on cholinesterase receptors (Caixeta et al., 2012).

In the case of less prevalent comorbidities, our data showed inconsistencies in data from Tables 3 and 5; most of the elderlies took antiparkinsonian medications (Table 3), however, Parkinson's disease was one of the least recurring among self-reported diseases (Table 5). This is due to the fact that the vast majority of our patients and caregivers had a low level of education (data

not shown), indicating the lack of information on drugs and their pharmacological actions in addition to the lack of attention or difficulty in understanding medical language. Laffa et al. (2013) observed this fact in their study where low education was significantly correlated with noncompliance with dosage ($p = 0.009$) suggesting difficulties related to the identification of drugs and administration techniques.

Cholinesterase inhibitors (IChE) are frequently used for the treatment of mild to moderate stages of AD (Forlenza, 2005; Fagherazzi et al., 2009). The prevalence of the use of the long-term medication, donepezil hydrochloride, was observed within this pharmaceutical class. Montastruc et al, (2013) also showed a high prevalence of donepezil use in a study that evaluated 684 elderlies: 610 of these were using some anticholinesterasic and, among these, 63% (n = 431) used donepezil. The prescription of this drug is quite frequent because this cholinesterase inhibitor presents low risks as compared to other IChEs; furthermore, it's the selectivity of donepezil results from prevention of peripheral side effects recurrent of its activity, its action time, and because its use is reversible preventing major risks in the event of poisoning (Lima, 2008).

Goes et al. (2015) assessed the nutritional status of a group of patients evaluated in the same city as this study and verified that most of the patients were using Donepezil and were in a state of malnutrition with low albumin levels. Because this drug requires interaction with albumin, the level of free molecules increases in patients with low albumin levels; these free molecules interact with other drugs such as some with cardiovascular activity.

Out of the 41 patients diagnosed with AD (in this study), only 34 (82.93%) use anticholinesterasic inhibitors. According to the Ministry of Health (2010) in the public consultation No. 15 of March 31, 2010, only patients with CDR equal to or less than 2 are entitled to free medication for care continuity at the Unified Health System (SUS) in Brazil. This medication withdrawal is justified by the fact that, even with the pharmacological approach being only symptomatic, the medication is ineffective due to the progressive degenerative characteristic of the disease (Forlenza, 2005). However, there are studies that indicate that the quality of life of the elderly in this scenario would be compromised because the degenerative disease processes accelerate the mental and functional decline, affecting the quality of life of patients and caregivers, even in the most advanced stage of AD (Inouye et al., 2010).

Hence, it is concluded that polypharmacy is present in the daily life of the elderly population with AD, and in greater numbers in populations with some type of NTD. However, this fact can lead to harmful consequences such as drug interactions, iatrogenic and adverse reactions among others. It was also evidenced that some of the studied elderlies make use of inappropriate drugs, either due to inattention or lack of medical knowledge.

Further studies investigating the interactions caused by these drugs and the consequences of these interactions with the patients will significantly contribute to preventing the occurrence of polypharmacy. These studies could also reinforce the need for guidance and pharmaceutical care on such drugs which could increase the awareness in the population of caregivers.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of the antioxidant activity of the leaves, stem-barks extracts and fractions of *Ochna schweinfurthiana* F.Hoffm (Ochnaceae)

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The present study evaluates the *in vitro* antioxidant activity of the leaves and stem-barks extracts and fractions of *Ochna schweinfurthiana*. To this effect, the different extracts were obtained by maceration in four solvents namely ethyl acetate, methanol, acetone and water- ethanol mixture (20-80). The methanol extract which exhibited the best antioxidant activity was partitioned in hexane and ethyl acetate. The ethyl acetate fraction was fractionated by column chromatography with the aid of methyl dichloride/methanol (CH_2Cl_2/ MeOH) solvent system at different polarities. The antioxidant activity of the extracts and fractions was assessed by the 2,2-diphenyl-1-picrilhidrazil (DPPH), ferric reducing antioxidant power assay (FRAP) and the total polyphenol content was evaluated using the Folin-Ciocalteu reagent. The results were analyzed using SPSS 20 presented as mean $\pm$ standard deviation. The results phytochemical screening confirmed the abundance of flavonoids and catechic tannins in the methanol and water-ethanol extracts whose content vary between 37.83 ± 1.6 mg ascorbic acid equivalent (EAA)/g dry weight (dw) and 96.4 ±2.33 mg EAA/g dw. The leaves methanol extract possess the best antiradical power (AP) of 0.00114 ± 0.00001 g/mg and the best ferric reducing antioxidant power (542.33±16.51 mg EAA/g dw). The F3 fraction obtained using CH_2Cl_2/MeOH 5/1 elution system possess the best AP of 0.00125 ± 0.00001 g/mg identical to that of ascorbic acid (AP = 0.00125 ± 0.00002 g/mg) and the strongest ferric reducing antioxidant power (508.66 ± 18 mg EAA/g dw). A positive correlation between the two antioxidant tests and the polyphenols content was obtained. Thus, *Ochna schweinfurthiana* could be used by the population to prevent some diseases caused by oxidative stress, due to its high antioxidant effect.

Key words: *Ochna schweinfurthiana*, extracts, fractions, antioxidant activity.

INTRODUCTION

Nowadays, the scientific world is putting into evidence the tragic role of the uncontrollable role of the oxidative

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process induced by reactive oxygen species (ROS). Oxidative stress is defined as the disequilibrium between the biochemical processes of free radical (FR) production and those of antioxidant defenses in favour of free radical production (Sayre et al., 2008). These free radicals could react with a series of biological substrates such as DNA, proteins, lipids and carbohydrates. They are directly related to a number of diseases such as early ageing, cataract, acute respiratory distress syndrome, pulmonary oedema (Favier, 2003). An aggravation of the initial process of free radicals production causes more severe illnesses such as cardiovascular diseases, some type of cancer, diabetes, Alzheimer, rheumatism (Sas et al., 2007). Based on this reality, a reawakening of phytotherapy which produces an important quantity of bioactive molecules and which have the capacity to trap these free radicals is a largely exploited domain. In effect, natural antioxidants are involved in several research and a new approach towards the exploitation of secondary metabolites in general and polyphenols in particular in health as well as in the agro-food industry (Prior et al., 2005). Flavonoids which constitute an important class of these compounds are widely researched for their biological properties: Antioxidant, anti-inflammatory (Rahman et al., 2006), antiallergic and anticancerous agents (Viana et al., 2003). *O. schweinfurthiana* F.Hoffm is a tree or shrup which can be up to 4 m long is found in the tropical forests of Africa, America and Asia. In Africa, it is found in Guinea up to the North and South of Nigeria, Central Africa to Sudan, Uganda, Zimbabwe and Mozambique (Abdullahi et al., 2010). Besides being used as a decorative plant because of its multi-colored flowers (Burkill, 1997), it is used in traditional medicine in the North of Cameroon to treat malaria, erethism and typhoid fever. Likewise, in the North of Nigeria, *O. schweinfurthiana* is used to treat measles, typhoid fever and skin fungal infections. Literature review on this plant revealed that the antimicrobial effect of the methanol and acetone extracts of the leaves of this plant on some selected pathogens has been carried out (Abdullahi et al., 2010). But no previous antioxidant investigation on the extract and fraction of the plant has been reported**.** To this effect, the aims of this study were to search for new natural antioxidant molecules by evaluating the antioxidant properties of the extracts and fractions of *O. schweinfurthiana.*

MATERIALS AND METHODS

The botanical material commonly known in Cameroon as Sa'aboule in fulfulde, is made up of leaves and stem-barks. It was collected in January 2014 in Ngaoundere and identified by Mr. Nana Victor of the National Herbarium of Cameroon under the identification code: 40171HNC.

Methods of extraction of phenolic compounds

Within the framework of this study, the stem-barks and leaves were subjected to cold maceration (Prakash et al., 2005). The solvents were used in order of increasing polarity: ethyl acetate, acetone methanol, ethanol-water (80-20 v/v). To obtain the extracts, 200 g of the powdered stem-barks and leaves were soaked separately in 650 mL of pure ethyl acetate. After 48 h of maceration, the mixture was filtered. The filtrate was concentrated using a rotar vapor of the model Janke. To obtain the ethyl acetate extract, the maceration was repeated twice in order to maximize the yield. The residue obtained after the ethyl acetate maceration was dried for 24 h then used in the next extraction. The same procedure was repeated to obtain the acetone, methanol and ethanol-water extracts. The leaves methanol extract that exhibited the strongest antioxidant activity was partitioned using hexane and ethyl acetate. The ethyl acetate fraction was fractionated on column chromatography with methyl dichloride/methanol elution system at different polarities (Lhuillier et al., 2007). At the end of the fractionation, four major fractions were grouped and denoted F1, F2, F3 and F4 depending on the speed of the spots on the chromatographic plates.

Phytochemical screening

Phytochemical screening of the extracts to identify different families of bioactive compounds found in the extracts was carried out as described by Harbone (1998) and Sofowora (1993).

2,2-diphenyl-1-picrilhidrazil (DPPH) antiradical test

To prepare a standard solution of 2,2-diphenyl-1-picrilhidrazil (DPPH°), 10 mg of DPPH was dissolved in 25 mL of methanol (Brand-Williams et al., 1995). From this solution, 5 mL was taken and mixed with 45 mL of methanol. After preparing the different solutions, 1950 µL of the DPPH solution was pipetted into test tubes and 50 µL of each extract at different concentrations was then added to each test tube to a final volume of 2 mL per tube. All tests were carried out in triplicate in a dark room. The optical density was measured at a wave length of 515 nm using a spectrophotometer of the brand Jenway 6305, Germany after 120 min of incubation.

Test of the ferric reducing antioxidant power assay: FRAP

The ferric reducing antioxidant power assay (FRAP) is based on the reduction of the tripyridyltriazine ferric complex (Fe^{3+}-TPTZ) to the tripyridyltriazine ferrous complex (Fe^{2+}-TPTZ) in the presence of an antioxidant, 1950 µL of FRAP solution was pipetted into different test tubes, follow by 50 µL of extracts or fraction at different concentrations (Benzie and Strain, 1999). The tests were done in triplicate, and the mixture was incubated for 30 min in darkness. The optical density was measured at 593 nm using a spectrophotometer of the brand Jenway 6305, Germany. The FRAP solution was prepared as follows: 14.1 mg of TPTZ was diluted in 9 mL HCL at 40 mM then ferric chloride ($FeCl_2$) at 20 mM and acetate buffer 300 mM at P^H 3.6 mixed in the ratio of 1: 1: 10 respectively to form the FRAP solution.

Titration of the total polyphenol content by the Folin-Ciocalteu test

The total polyphenols was evaluated by spectrophotometry using Folin-Ciocalteu reagent as described by Chew et al. (2009). A volume of 1817 µL of distilled water was introduced in a test tube, 115 µL of Folin-Ciocalteu diluted at 1/10 and 345 µL of sodium carbonate(Na_2CO_3) at 15% were added. The tubes were well vortexed, incubated for 2 h and the absorbance read at 765 nm

Table 1. Summary of the phytochemical screening of the leaves and stem barks extracts of *Ochna schweinfurthiana.*

Tests	Ethyl acetate Ex.		Methanolic Ex.		Ethanol/water Ex.		Acetone Ex.	
	Stem-barks	Leaves	Stem-barks	Leaves	Stem-barks	Leaves	Stem-barks	Leaves
Flavonoids	-	+	++	++	+	+	+	+
C. Tannins	-	+	++	++	++	+	+	+
Steroids	-	++	-	+	-	-	-	+
Saponins	+	-	++	++	++	+	+	+
Alkaloids	-	+	+	+	+	+	+	+
Triterpenes	+	-	++	-	++	+	++	-

Presence (+); Absence (-); Abundance (++).

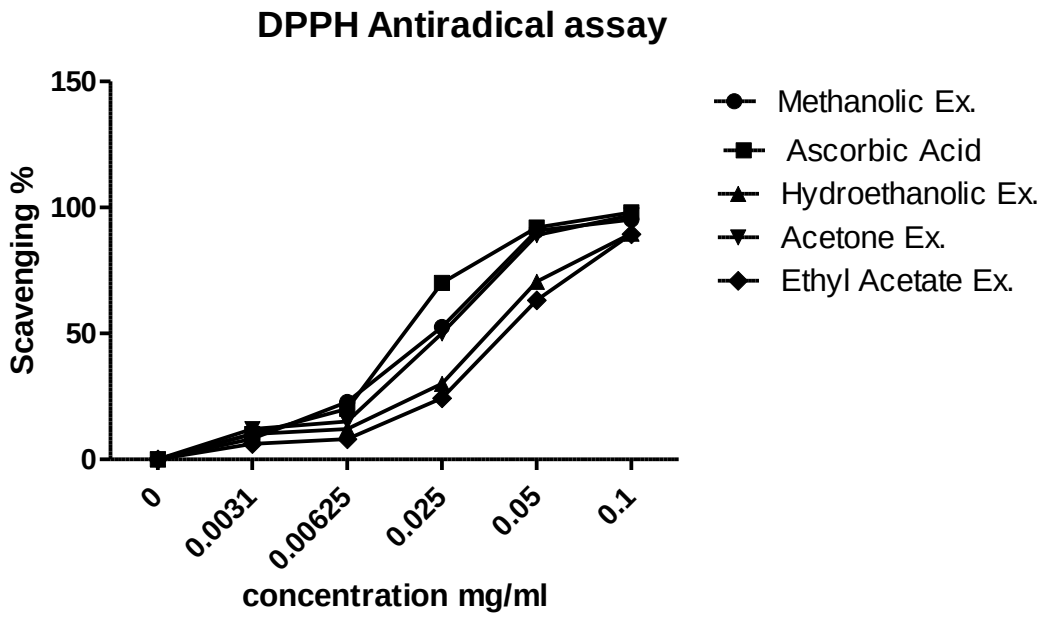

Figure 1. Variation curve of the DPPH scavenging percentage versus concentration of stem-barks extracts of *O. schweinfurthiana.*

using a spectrophotometer of the brand Jenway 6305, Germany. The standard solution was prepared using a freshly prepared aqueous solution of ascorbic acid.

RESULTS AND DISCUSSION

Phytochemical screening

Result of the phytochemical tests of the different extracts is shown in Table 1. The results indicated the abundance of flavonoids, saponins and catechic tannins in the methanolic extracts; the presence of alkaloids and triterpenes in other extracts.

Evaluation of the *in vitro* antiradical power by the DPPH test

Figures 1 to 3 show a general increase in the scavenging percentage of DPPH free radicals in all the extracts. On a general basis, the methanol extract of the leaves shows the greatest scavenging activity, followed by the hydro-ethanol extract of the leaves; the fraction F3 shows a high scavenging activity followed by the F4 fraction. All these active extracts and fractions have a better hyperbolic curve than that those of the extracts and fractions exhibiting low scavenging activity.

Results of the antiradical DPPH test carried out on *O. schweinfurthiana* extracts and fractions are shown in Table 2. The methanolic extract of leaves and fraction F3 show the greatest antiradical power (AP) of 0.00114 ± 0.00001 g/mg and 0.00125 ± 0.00001 g/mg, respectively.

Evaluation of the *in vitro* reducing power by the FRAP test

Figures 4 to 6 show the variation of the different extracts

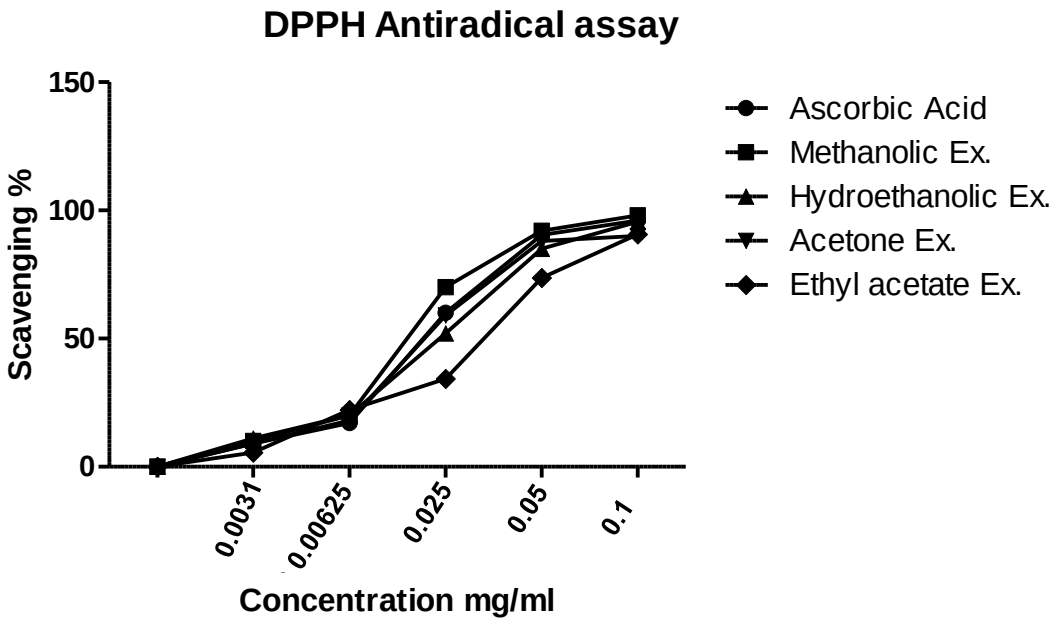

Figure 2. Variation curve of the DPPH scavenging percentage versus concentration of leaves extracts of *O. schweinfurthiana*.

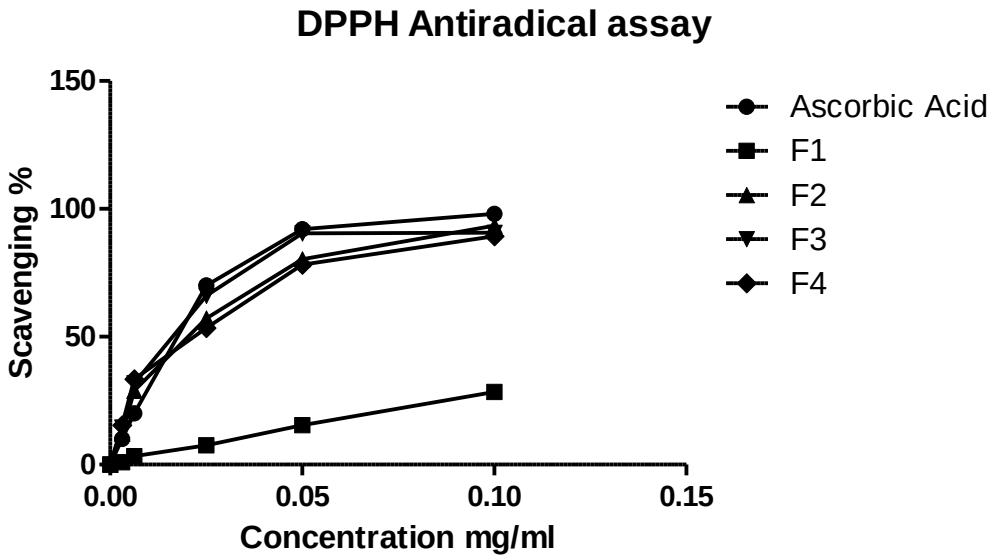

Figure 3. Variation curve of DPPH scavenging percentage versus the fractions concentration of *O. schweinfurthiana*.

in FRAP. The reducing power in mg ascorbic acid equivalent (EAA)/g dry weight (dw) was evaluated using the regression line of the optical density variation versus concentration of the extracts, fractions and reference molecule. In Table 3, it is seen that methanol extract of leaves, hydroethanolic stem-bark extract, fraction F3 and fraction F4 have the highest capacity to reduce ferric ions.

Titration of the total polyphenols and correlation with the antioxidant activity of the extracts and fraction

Table 4 summarizes the antiradical power, the reducing power and the total polyphenols content of the different extracts and fractions. The results of the titration of the total polyphenols show that the extracts and fractions exhibiting the strongest antioxidant activity contain high

Table 2. Summary of the antiradical activity assessment of the leaves and stem barks extracts of *Ochna schweinfurthiana.*

Tested substances	SC_{50} (g/l)	CE_{50} (mg Ex/g of DPPH)	AP (g/mg)
Ascorbic acid	0.032 ± 0.001	800 ±15	0.00125 ± 0.00002^{e}
Methanol Leaves Ex.	0.035 ± 0.001	875 ± 14	0.00114 ± 0.00001^{de}
Acetone leaves	0.037 ± 0.002	925 ± 16	0.00108 ± 0.00001^{d}
Ethanol-water leaves Ex.	0.0371± 0.002	928 ± 15	0.00107 ± 0.0001^{d}
Ethyl acetate leaves Ex	0451 ± 0.0019	1127.5 ± 14.3	$8.86x10^{-4}\pm0.0000^{c}$
MeOH stem-barks Ex.	0.036 ± 0.001	900 ± 17	0.00111 ± 0.00001^{de}
Acetone stem-barks Ex.	0.036 ± 0.001	900 ± 17	0.0011 ± 0.0001^{d}
Ethanol-water stem-barks Ex.	0.038 ± 0.001	950 ± 15	0.00105 ± 0.00001^{d}
Ethyl acetate Stem-barks Ex.	0.0504 ± 0.002	1600 ± 16	$7.93x10^{-4}\pm0.0000^{c}$
Fraction F1	0.175 ± 0.007	4375 ± 19	$2.28x10^{-4}\pm0.0000^{b}$
Fraction F2	0.0359 ± 0.0001	897.5 ± 12.5	0.001 ± 0.000^{d}
Fraction F3	0.031 ± 0.001	799.87 ± 6.84	0.00125 ± 0.00001^{e}
Fraction F4	0.036 ± 0.002	900 ± 8	0.0011 ± 0.0001^{d}

Values with the same letters are statistically identical meanwhile those with different letters are statistically different with a threshold value of $P < 0.05$.

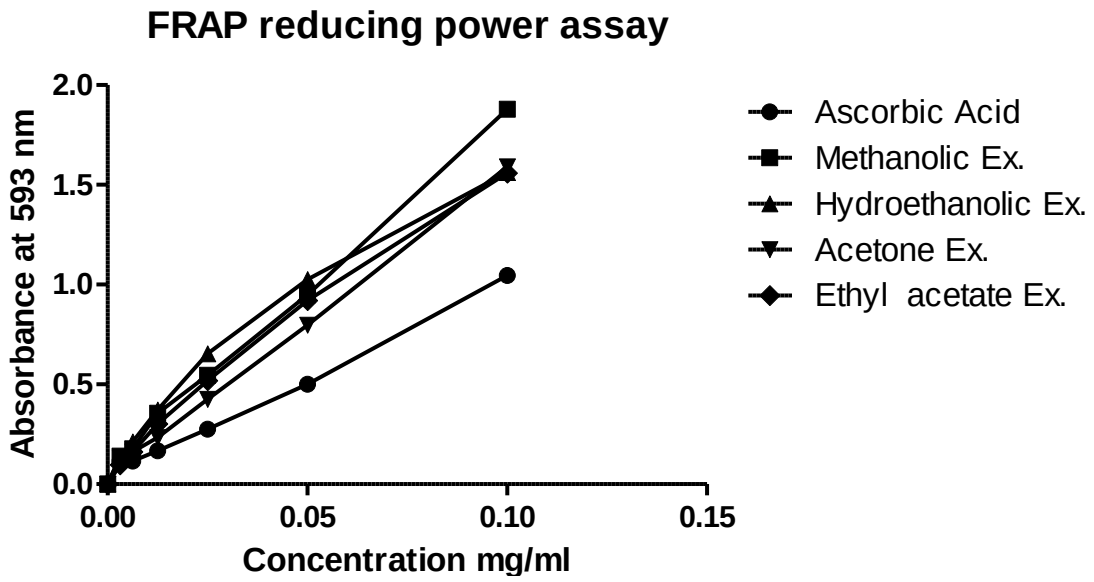

Figure 4. Absorbance variation curve of leaves extracts of *O. schweinfurthiana* and ascorbic acid versus concentration.

amounts of polyphenols.

In Figures 7 and 8, the correlation between the scavenging activity (DPPH), the antioxidant activity (FRAP) and the polyphenol content is positive ($R^2 = 0.95$; $R^2 = 0.91$ respectively) revealing that antioxidant activity depends on the polyphenol content.

DISCUSSION

The phytochemical screening carried out on the extracts of *O. schweinfurthiana* indicates the presence of catechic tannins, triterpenes, alkaloids, saponins and flavonoids. All this group of compounds has been reported by Abdullahi et al. (2010) in acetone and methanol extracts of *O. schweinfurthiana*.

To the best of our mind, the antioxidant activity of *O. schweinfurthiana* has not been evaluated. However, results of the DPPH antiradical assay confirm the phytochemical screening carried out on the different extracts. In effect, the antiradical power of the different extracts and fractions was determined by the DPPH assay. It is noticed that the extracts that exhibit a weak antiradical activity with respect to ascorbic acid (AP of

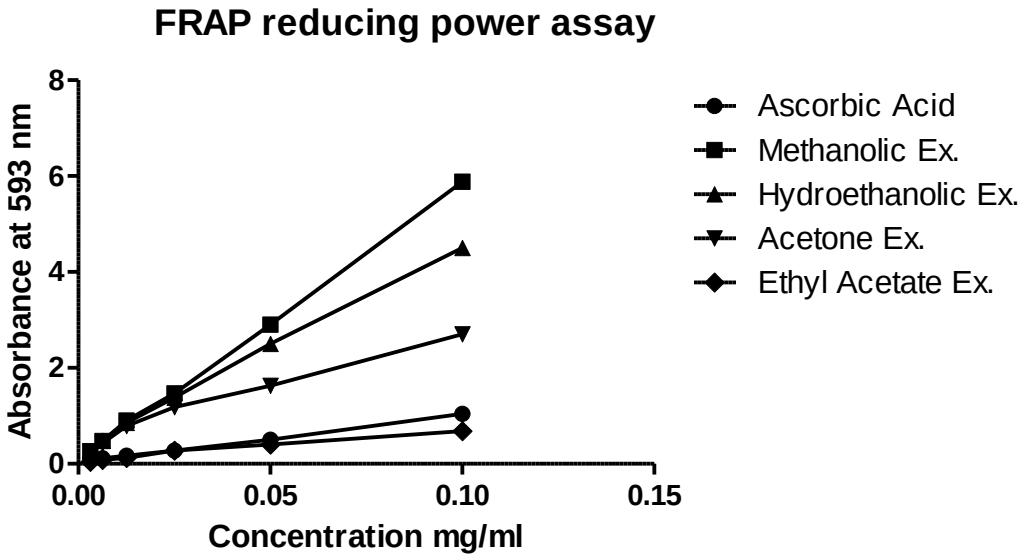

Figure 5. Absorbance variation curve of stem-barks extracts of *O. schweinfurthiana* and ascorbic acid versus concentration.

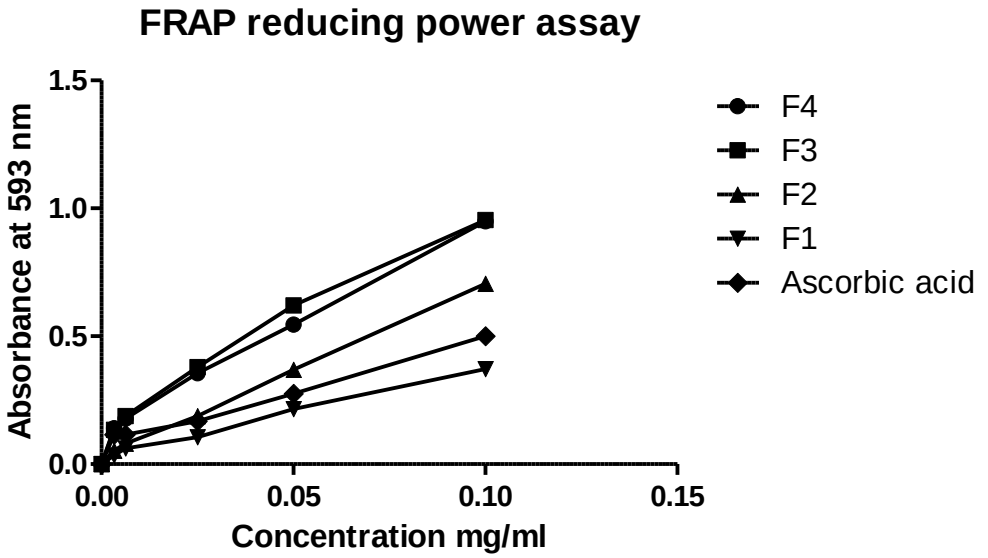

Figure 6. Absorbance variation curve of the fractions of *O. schweinfurthiana* and ascorbic acid versus concentration.

0.00125 ± 0.0002 g/mg) are those obtained using fairly polar extraction solvents. They are the leaves and stem-barks ethyl acetate extract, which have an AP of $8.86x10^{-4}$ ± 0.0000 g/mg and AP of $7.93x10^{-4}$± 0.0000 g/mg respectively. This could be explained by the fact that the extracts are made up of less polar compounds which have a weak antioxidant activity (Koffi et al., 2010). Acetone and ethanol-water extracts statistically have the same antiradical activity. Their antiradical power vary between AP of 0.00105 ± 0.00001 g/mg and AP of 0.00107 ± 0.00001 g/mg. Methanol leaves and stem-barks extracts exhibited the highest AP of 0.00111± 0.00001 and AP of 0.00114 ± 0.000001 g/mg respectively. Nevertheless, they possess an antiradical activity weaker than that of ascorbic acid AP of 0.00125 ± 0.00002 g/mg. This result is confirmed by the phytochemical screening which revealed that the methanol extracts contained much polyphenols and flavonoids which by themselves possess high inherent antiradical activity (Crozier et al., 2006).

Table 3. Summary results of the FRAP assay

Tested substances	Reducing power (mg EAA/g dw)
Methanol leaf Ex.	542.33 ± 16.51^{h}
Ethanol-water leaf Ex.	242.66 ± 4.52^{f}
Acetone leaf Ex.	238.33 ± 3.64^{e}
Ethyl acetate leaf Ex.	236 ± 6^{f}
MeOH stem-barks Ex.	513 ± 14^{g}
Ethanol-water stem-barks Ex.	529.33 ± 15.01^{g}
Acetone stem-barks Ex.	243 ± 2^{f}
Ethyl acetate stem-barks Ex.	67.16 ± 1.8^{c}
Fraction F1	50.96 ± 3.4^{b}
Fraction F2	89.51 ± 2.5^{d}
Fraction F3	508.66 ± 18^{g}
Fraction F4	502.33 ± 10.41^{g}

Values with the same letters are statistically identical meanwhile those with different letters are statistically different with a threshold value of $P < 0.05$.

Table 4. Antiradical power, reducing power and quantity of total polyphenols of *Ochna schweinfurthiana.*

Tested substances	Antiradical power (g/mg)	Reducing power (mg EAA/g dw)	$Q_{polyphenols}$ (mg EAA/g dw)
Methanol leaf Ex.	0.00114 ± 0.00001^{de}	542.33 ± 16.51^{h}	96.4 ± 2.33^{i}
Ethanol-water leaf Ex.	0.00108 ± 0.00001^{d}	242.66 ± 4.52^{f}	3783 ± 1.6^{cde}
Acetone leaf Ex.	0.00108 ± 0.00001^{d}	238.33 ± 3.64^{e}	48 ± 2^{bcd}
Ethyl acetate leaf Ex.	$8.86x10^{-4} \pm 0.0000^{c}$	236 ± 6^{f}	28.98 ± 1.1^{bcd}
MeOH stem-barks Ex.	0.00111 ± 0.00001^{de}	513 ± 14^{g}	62.66 ± 6.25^{efg}
Ethanol-water stem-barks Ex.	0.00105 ± 0.00001^{d}	529.33 ± 15.01^{g}	75.9 ± 4.1^{hi}
Acetone stem-barks Ex.	0.0011 ± 0.0001^{d}	243 ± 2^{f}	75.9 ± 4.1^{hi}
Ethyl acetate stem-barks Ex.	$7.93x10^{-4} \pm 0.0000^{c}$	67.16 ± 1.8^{c}	28.93 ± 1.3^{bcd}
Fraction F1	$2.28x10^{-4} \pm 0.0000^{b}$	50.96 ± 3.4^{b}	22.6 ± 3.5^{abc}
Fraction F2	0.001 ± 0.000^{d}	89.51 ± 2.5^{d}	24.46 ± 2.7^{abc}
Fraction F3	0.00125 ± 0.00001^{e}	508.66 ± 18^{g}	73.43 ± 1.9^{ghi}
Fraction F4	0.0011 ± 0.0001^{d}	502.33 ± 10.41^{g}	53.4 ± 6.8^{defg}

Values with the same letters are statistically identical meanwhile those with different letters are statistically different with a threshold value of $P < 0.05$.

Similarly, this study reveals that the antiradical activity of the extracts and fractions is due to the polarity of their constituting compounds. Faction F1 and F2 obtained using weak polar solvents (CH_2Cl_2/MeOH 50/1 to CH_2Cl_2/MeOH 35/1) exhibited weak AP of $2.28x10^{-4}$ ± 0.0000 g/mg and AP of 0.001 ± 0.000 g/mg respectively. Fraction F3 was obtained using the CH_2Cl_2/MeOH 5/1 solvent system and shows an AP of 0.00125 ± 0.00001 g/mg equivalent to that of ascorbic acid while fraction F4 obtained from the CH_2Cl_2/MeOH 1/1 solvent system gave an AP of 0.0011 ± 0.0001 g/mg lower than that of fraction F3. This could be explained by the fact that the antiradical activity is strongly associated to the chemical structure of the compounds responsible for such an activity and the synergistic or antagonistic effects of the different compounds present in the fraction which could increase or decrease its antiradical activity (Frankel, 1998).

It is equally noticed that, the reducing power of the extracts and fractions is strongly related to the polar nature of the compounds that make it up. In effect, the ethyl acetate extracts have low reducing powers. Their ascorbic acid equivalent concentration is between 67.16 ± 1.8 and 236 ± 3 mg EAA/g dw for the leaves and stem-barks respectively. The acetone, ethanol-water and methanol extracts have a strong reducing power and their ascorbic acid equivalent concentration is comprised between 238.33 ± 3.64 and 542.33 ± 16.51 mg EAA/g dw, respectively. These results are in harmony with those found in literature. In effect it has been demonstrated that

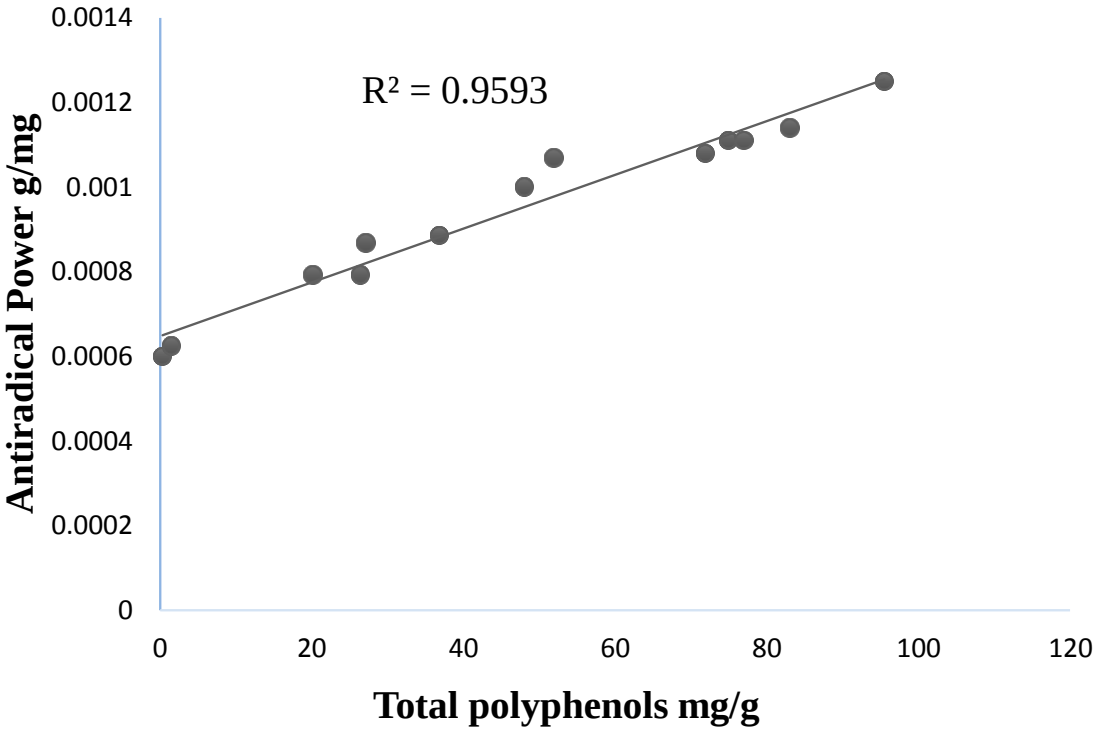

Figure 7. Correlation between antiradical activity of the extracts and fractions of *Ochna schweinfurthiana* and total polyphenols content.

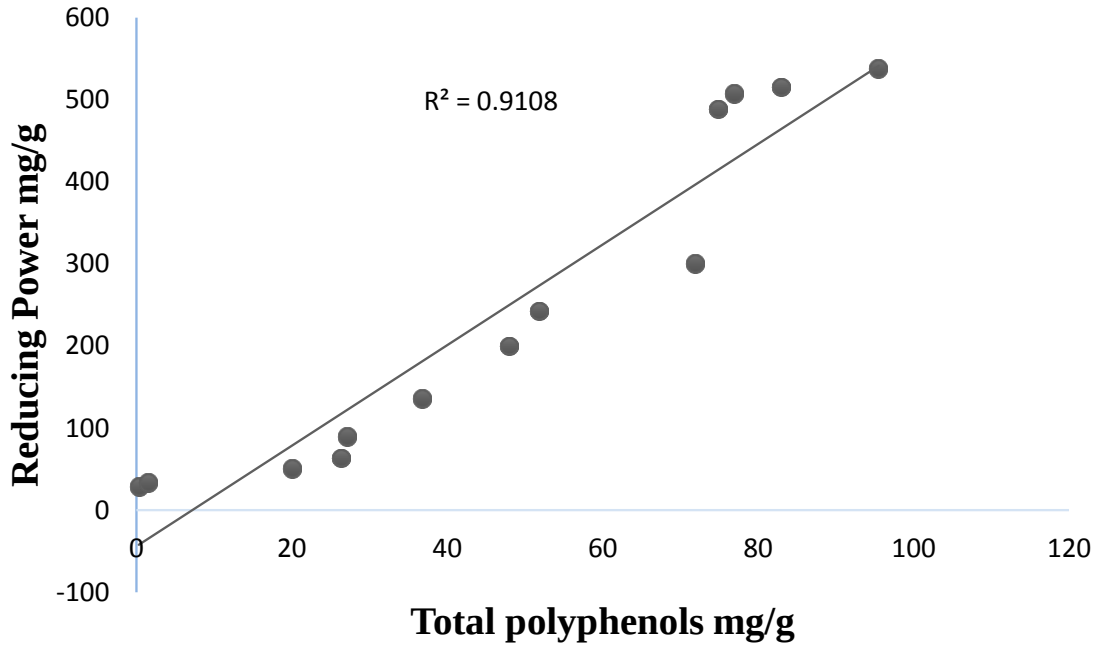

Figure 8. Correlation between the reducing power of the extracts and fractions of *Ochna schweinfurthiana* and total polyphenols content.

phenolic compounds most especially flavonoids by virtue of their chemical structure possess a strong reducing power (Crozier et al., 2006). The reducing power of the fractions varies between 50.96 ± 3.4 and 508.66 ± 18 mg EAA/g dw for fractions F1 and F3, respectively.

Titration of polyphenols in the different extracts and

fractions ascertain their antioxidant activity. In effect, the least active extracts and fractions are those with low polyphenol content (stem-barks ethyl acetate extract and fraction F1) equal to 28.93 ± 1.3 and 22.6 ± 3.5 mg EAA/g dw, respectively, meanwhile the most active extracts and fractions were the leaves methanol extracts and fraction F3 with have a polyphenol content of 96.4 ± 2.33 and 73.43 ± 1.9 mg EAA/g dw, respectively. The correlation between the antiradical activity, the reducing capacity of the extracts and fractions with the polyphenol content is very high. Thus, the correlation coefficient between the antiradical activity and the polyphenol content is R^2 = 0.95 and that between the reducing capacity and polyphenol content is R^2= 0.91.

Conclusion

At the end of this study which aimed at searching for new sources of natural antioxidants by assessing the antioxidant properties of the extracts and fractions of the leaves and stem-barks of *O. schweinfurthiana*, the study reveals that this plant possesses a good antioxidant activity. *O. schweinfurthiana* is very rich in polyphenols such as flavonoids and catechic tannins. The leaves methanol extract and fraction F3 which was obtained using CH_2Cl_2/MeOH (5/1) solvent system gave the strongest antioxidant activity. The antioxidant activity is due to the presence of polyphenol compounds. The correlation coefficient between the antiradical activity and the total polyphenol content is R^2=0.95 and that between the reducing power and the total polyphenols is R^2= 0.91. The results of this study rationalize the ethno-medicinal use of *O. schweinfurthiana*.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Larvicidal potential of *Mikania glomerata* SPRENGEL extract on *Ancylostoma caninum* larvae

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Parasitic diseases are seen as indicators of a country's socioeconomic development, constituting a major public health problem as they cause direct health problems related to the lack of piped water, no sewage system, and lack of orientation. Contamination by the geohelminth *Ancylostoma* spp, causes the Cutaneous larva migrans (CLM), also known as "sandworms", presenting skin lesions of linear and serpiginous character. The aim of this study was to evaluate the *in vitro* larvicidal potential of guaco extracts (*Mikania glomerata* SPRENGEL) at different concentrations on *A. caninum* larvae. Obtained results showed the larvicidal effect of the *M. glomerata* extract starting from a treatment of 10mg/ml of guaco extracts ($p<0.01$). The larvicidal activity was best demonstrated in the 25 mg/ml treatment, in which a decrease of 13.30% of L3 was observed compared to the control group, and in the 50 mg/ml treatment (61.66%) reduction of L3. By means of the results, the applicability of the plant extracts used is suggested in *A. caninum* larvae control. In addition, more research is suggested to assess their employability in different extract forms, new concentrations, and *in vivo* studies, in order to ensure further clarification on the agents responsible for the observed effects, degree of efficacy and toxicity, and research continuity regarding the use concentration of the plant *M. glomerata* SPRENGEL.

Key words: Larvicidal extract, *Mikania glomerata*, *Ancylostoma caninum* and cutaneous larva migrans.

INTRODUCTION

Currently, due to the presence of cases with resistance to anthelmintic drugs and the need for new approaches to nematode control consisting of great zoonotic potential, there has been a resurgence of research on substances with natural anthelmintic properties. Plants with "popular" use are most often the material studied and, this line of research has been encouraged mainly by the fact that such plants have been traditionally used by indigenous peoples, particularly in the tropics, against gastrointestinal nematodes of both humans and animals (Stepek et al., 2006). In this regard several studies using medicinal plants and its derivatives have shown ovicidal

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and larvicidal activity against various parasites (Lone et al., 2012; Bi and Goyal, 2012; Sousa et al., 2013).

The *Mikania glomerata* SPRENGEL., is a Brazilian native plant belonging to the Asteraceae family, made official in the 1st edition of the Brazilian Pharmacopoeia. Despite not having its chemical composition fully elucidated the *M. glomerata* Sprengel is one of the most studied species in the pharmacognostic aspect. For *M. glomerata* several pharmacological activity were observed, including antifungal action, antimicrobial, bronchodilator, anti-allergic, anti-inflammatory (Brandão et al., 2006; Amaral et al., 2009; Celeghini et al., 2001).

The soil and public parks is via transmission to parasitic zoonosis. One of the most frequent is the *Ancylostoma* spp, one geohelminth parasite that dogs and cats and, possibly affects human beings, causing larva migrans skin (CML) (Santarem et al., 2004). Faced with the gastrointestinal parasite *A. caninum* other plants were evaluated and the sensitivity of these parasites to the *Carica papaya* L. extract was observed, suggesting a potential use of the plant as an anthelmintic against *A. caninum* infection in mice (Lone et al., 2012; Bi and Goyal, 2012).

Thus, the potential of medicinal plants and their derivatives as ovicides and or larvicides is clear and there are a various different plant species which have not yet been evaluated as for this activity. *A. caninum* infection occurs by ingestion or skin penetration of infective larvae (L3), its penetration is mainly performed through the skin of the lower limbs. Through blood circulation, they reach the pulmonary capillaries, traverse the alveolar wall, ascend with mucous secretions from the bronchial tree to the larynx and pharynx and are swallowed, make it to the intestine, where the last changes and the final transformation in adult worms, male and female occurs, being necessary measures to prevent contamination (Rey, 2001).

New therapeutic approaches are also essential for the control of parasites responsible for zoonoses. Some alternative measures are needed to assist in soil decontamination. One alternative that has been evaluated is the use of herbal medicines and their derivatives (extracts, enriched fractions, essential oil, dye) in the control of gastrointestinal parasites. This measure, in addition to having been proven effective in several studies, has the advantage of being sustainable and not damaging the environment. Thus, the objective of this study was to evaluate the *in vitro* larvicidal potential of guaco extracts (*M. glomerata* SPRENGEL) at different concentrations on *A. caninum* larvae.

MATERIALS AND METHODS

Ethical aspects

This project was approved by the Ethics Committee on Animal Use (CEUA- UVV) University of Vila Velha (UVV-CEUA), which opinion is embodied No 292/2013.

Plant material

The crude extract of the plant *M. glomerata* SPRENGEL was provided by the Medicinal Plant Industry of the Department of Agriculture of the Federal University of Lavras - MG (DAG / UFLA). The plant was identified by Dr. Mara Ritter of the Institute of Biosciences, Federal University of Rio Grande do Sul, where the evidence samples (herbarium specimens) are deposited under registration number ICN 141992. The dried *M. glomerata* plant material (260 g) was submitted to percolation with 96° GL ethanol. The ethanolic extract was concentrated in a rotary evaporator at 50°C under reduced pressure, obtaining 55 g of *M. glomerata* ethanol extract.

Extract preparations

Extract concentrations were prepared as described in the 3rd edition of the Brazilian Homeopathic Pharmacopoeia. The crude extract was weighed on an analytical scale and added solvent ethanol 96.5 GL° and taken to ultrasonic ultrasound. The preparation was maintained protected from direct light and heat and hermetically sealed.

Obtaining of *Ancylostoma caninum* larvae

Fresh feces from dogs living in the city of Vitória - ES, Southeastern Brazil, were collected and from these fecal samples about 3 to 5 g of feces were taken for performance of the fecal flotation technique (Willis-Mollay technique) to analyze if there was presence *A. caninum* eggs. After identification of *A. caninum* eggs, fecal cultures were prepared with about 20 g of feces mixed with autoclaved industrial vermiculite and moistened with water, the larval cultures were incubated in a BOD chamber during a period of 7 days. After this period the 3rd stage larvae were extracted and identified by the Baermann technique and quantified in an optical microscope and 10x objective.

Experimental assay

The experimental trial aimed to analyze the larvicidal activity of the extracts at four different concentrations (1, 10, 25, and 50 mg/ml) on *A. caninum* infective larvae (L3). The testing was performed in monofactorial experiment with: 1. CW; 2.CE; 3. T1 mg/ml; 4. T10 mg/ml; 5. T25 mg/ml; 6. T50 mg/ml.

The larvicidal activities of the *M. glomerata* ethanol extract in concentrations, 1; 10; 25 and 50 mg/ml, and ethanol on *A. caninum* cultures and a control group without treatment (water), were evaluated. For this, Petri dishes of 9.0 cm in diameter with 6 ml of 2% agar medium were prepared with about 1000 *A. caninum* larvae in each plate, and added 1 mL of each concentration of the *M. glomerata* extract and control group (1 ml ethanol solvent used for preparation of extracts) and a group without treatment (1 ml of water). Each treatment consisted of three replicates. During the seven-day period the plates remained in the conservatory and every 24 h, 10 random fields of 4 mm were observed daily under a light microscope with 10x objective, and the number of larvae were counted in each field. At the end of seven days, *A. caninum* larvae were recovered from the content in the Petri dishes used in the experiment by the Baermann method (Lopes et al., 2015).

Statistical analysis

The data were interpreted statistically by analysis of variance at significance levels of 1 and 5% probability (Ayres et al., 2003). The

L_3 destruction efficiency compared to the control was evaluated by the Tukey test at 1% probability, with BioEstat 5.0. Later the larvicidal ability of the extracts was determined by the reduction percentage, using the following formula:

$$\text{Larval \% Reduction} = \frac{\text{Average larvae } (L_{3)} \text{ recovered from control - Average larvae } (L_{3)} \text{ recovered from treatments x100}}{\text{Average larvae } (L_{3)} \text{ recovered from control}}$$

RESULTS AND DISCUSSION

The pharmacognostic analysis of the crude extract of *M. glomerata* presented moisture content of (8.4 ± 0.2% g/g) and total ashes equal to (2.2 ± 0.2% g/g). The extractable matter demonstrated ethanol yield of (47.33 ± 2.1% g/g). The pharmacognostic analysis presented moisture and ash within the limits described in the Brazilian Pharmacopoeia. The levels of coumarin in *M. glomerata* samples were detected, but at concentrations below the quantitation limit for the established method.

The presence of coumarin in *M. glomerata* samples was reported in several articles, but there are also articles reporting its absence, since this compound could not be quantified in the samples (Bertolucci et al., 2013). However, there are other metabolites described as a majority in the plant under study, like diterpenes, in particular the class of kauranes, present in the species *M. glomerata* have other pharmacological actions, particularly antiparasitic activity, and therefore further investigation is needed (Gasparetto et al., 2010). The total action of an extract is the sum of the activities of its constituents' (Lone et al., 2012). What corroborates with the results of this essay, where *M. glomerata* extracts showed some action on the L3 different from the control.

During 7 days the plates remained incubated and counting of 10 random fields from each plate was performed. After the seven days of the experiment, the L_3 were recovered by the Baermnan method using the material in the Petri dishes. The means and standard deviations for each test and recovery of larvae are shown in Table 1. However, it can be observed that there was no difference between the treatments, but during the intervals of days studied differences were noted between the treated groups and the control groups (C.W. and C.E). For example, on Day 1 there were difference controls (C.W. and C.E) over the tested concentrations. Another difference was noted in example C.E., the relative concentration of 1 to 10 mg /ml. Some literatures suggest that L3 may eventually escape to the periphery of the plates on agar plates (Eren and Pramer, 1965).

Comparing the mortality rates of the treatments and control groups, it was observed that the negative control (C.W- control with water) and positive control (C.E- control with ethanol) were not able to significantly reduce the number of L_3. Difference was also noted ($p < 0.01$) between treatments. However, in the last two treatments T25 and T50 mg/ml reduction ($p<0.01$) of L_3 was observed, suggesting that in greater concentrations the plant extract began to exert activity on L_3. Contributing to this study, researchers recorded that the anthelmintic activity of *Origanum vulgare* (Lamiaceae) dye observing that the capacity reduction was also directly related to the concentration of the extract (Dias de Castro et al., 2013).

The T1 and T10 mg/ml showed differences ($p < 0.01$) compared with the C.W and C.E, but the average of the C.E (4.9 ± 3.06) was lower than in T1 mg/ml (12 63 ± 8.75) and T10 mg/ ml (12.26 ± 8.49), after the second day the T1 and T10 mg/ ml groups showed differences when compared to the control groups ($p < 0.01$). On the sixth day of the experiment the T10 mg/ml does not differ from T25 mg/ml, suggesting that in this time period the T10 mg/ml demonstrates its greatest effect. On the last day of the experiment the treatments showed a difference between concentrations T1 and T10 mg/ml ($p< 0.01$).

The extract's activity increased with interaction time and was higher in concentrations above T10 mg/ml. In a study with the aqueous extract *Morinda citrifolia* no effect was observed at lower concentrations used and after 48 h at a concentration of 26.96 $mg.ml^{-1}$, this effect not being statistically significant when compared to the negative control ($p>0.05$). However, in hours 72 and 96 at concentrations of 13.48 and 26.96 $mg.ml^{-1}$, there was a difference, considering the aqueous extract of *M. citrifolia*, the positive control and the negative control (water). Comparing the mortality rates of the treatment and the negative control, it is observed that in the last two periods of time, there is a greater discrepancy of the effectiveness of the *M. citrifolia* aqueous extract in relation to the test with water (Brito et al., 2009).

During the seven days of interaction statistical difference between treatment groups was noted, not being demonstrated only between T25 and T50 mg/ml, suggesting that these concentrations the larvicidal effect demonstrated action stability. This feature is also observed in other studies on anthelmintic activity (Lone et al., 2012; Dias de Castro et al., 2013; Brito et al., 2009; Santana et al., 2013).

During the experiment in some days the average of the treatments was higher than the control, as on day 2, where T 1 mg/ml showed (20.6 ± 6.66%) and C.W (5.4 ± 2.58%) which can be explained due to the count of 10 daily fields being random on the Petri dish, not being chosen a field with larva so as not to induce the experiment results. Another finding that contributes to this fact is the characteristic of L3 migration to the middle of

Table 1. Means and standard deviations for each test and recovery of larvae during 7 days.

Test	Day 1	Test	Day 2
	Mean (%) and standard deviation		Mean (%) and standard deviation
C.W	9^{A} ±4.17	C.W	5.4^{A} ±2.58
C.E	4.9^{A} ±3.0	C.E	4.6^{A} ±1.83
T 1 mg/mL	12.6^{ABA} ±8.75	T 1 mg/mL	20.6^{BA} ±6.66
T10 mg/mL	12.3^{ABCB}±8.49	T10 mg/mL	13.73^{BAC} ±7.31
T25 mg/mL	2.6^{BCB}±1.83	T25 mg/mL	4.46^{AABC}±3.06
T50 mg/mL	1.5^{BAABC}±1.01	T50 mg/mL	2.00^{AABC}±0.75
	Day 3		**Day 4**
C.W	2.4^{A} ±1.06	C.W	3.85^{A} ±1.70
C.E	3.0^{A} ±1.36	C.E	2.42^{A} ±1.55
T 1 mg/mL	14.0^{BA} ±6.40	T 1 mg/mL	7.57^{BA} ±5.73
T10 mg/mL	6.13^{BAC} ±2.38	T10 mg/mL	6.35^{AB} ±3.43
T25 mg/mL	3.0^{AABC}±1.19	T25 mg/mL	2.14^{ABC}±1.16
T50 mg/mL	1.2^{AABC}±0.41	T50 mg/mL	1.64^{ABC}±0.74
	Day 5		**Day 6**
C.W	1.76^{A} ±0.833	C.W	1.5^{A} ±0.52
C.E	2.38^{A} ±1.70	C.E	1.3^{A} ±0.48
T 1 mg/mL	8.0^{BA} ±3.5	T1 mg/mL	9.4^{BA} ±3.59
T10 mg/mL	6.23^{BAAC} ±3.70	T10 mg/mL	5.8^{BACA} ±3.96
T25 mg/mL	1.84^{AABC}±0.68	T25 mg/mL	3.1^{AABC}±1.72
T50 mg/mL	0.534^{AABA}±0.51	T50 mg/mL	0.1^{AABA}±0.31
	Day 7		**Recovery of larvae**
C.W	2.0^{A} ±0.47	C.W	5.28^{A} ±2.62
C.E	1.6^{A} ±0.6	C.E	3.85^{A} ±1.46
T1 mg/mL	5.3^{BA} ±2.,62	T1 mg/mL	7.71^{AB} ±2.87
T10 mg/mL	5.4^{BAA} ±4.45	T10 mg/mL	2.71^{AABA} ±1.11
T25 mg/mL	1.3^{AABC}±0.48	T25 mg/mL	0.85^{BACA}±0.37
T50 mg/mL	0.6^{AABC}±0.51	T50 mg/mL	1.85^{BACA}±0.37

Means followed by the same lower case letters (column) do not differ statistically (p>0.01). Tukey Test.

the dish or its extremities, thus demonstrating that the L3 are active, also observed in another study (Lopes et al., 2015). Therefore the completion of the L3 recovery after 7 days of interaction is necessary to verify reduction percentage in each group. After the seven days of the experiment, the L3 were recovered by the Baermnan method using the material in the Petri dishes. The means and standard deviations of each test are shown in Figure 1. At the end of the experiment, the T1 mg/ml average (7.71 ± 2.87%) was greater than the mean in the ethanol control group (C.E) (3.85± 1.46%) and control with water (C.W) (5.28± 2.62%), a fact also observed by Lopes et al. (2015), being common in experiments with larvae, as they migrate to the center or extremities of the dish, location with more moisture.

In the T10 mg/ml (2.71 ± 1.11%); T25 mg/ml (0.85± 0.37%) and T50 mg/ml (1.85 ± 0.37%) groups the average of L3 recovered was lower than the average in the ethanol control group (CE) and control with water (CW), with a significant difference ($p < 0.01$). In a study with *Haemonchus contortus* larvae and eggs, the larvae and eggs were submitted to contact of four distinct extracts of hexane, chloroform, ethyl acetate, and methanol at five different concentrations (3.1, 6.2, 12.5, 25.0, and 50.0 mg/ml) from the plant *Spigelia anthelmia*. At a concentration of 50.0 mg/ml the ethyl acetate extract inhibited 100% of the eggs hatched and 81.2% of larval development. Similarly the methanol extract inhibited 97.4% of the hatching eggs and 84.4% of *H. contortus* larvae in development, while the other extracts showed lower percentages or even statistically identical to the control (Assis et al., 2003).

It is suggested that the larvicidal effect of *M. glomerata* extract was established starting at T10 mg/ml. Authors when studying the anthelmintic effect of *Euphorbia helioscopia* L., in the form of aqueous solution and methanolic extract observed that the *E. helioscopia* L. aqueous extract did not reduce egg count in the feces, *in vitro* studies showed increased nematode motility (98%) in higher concentrations of methanol extract (50 $mg.ml^{-1}$)

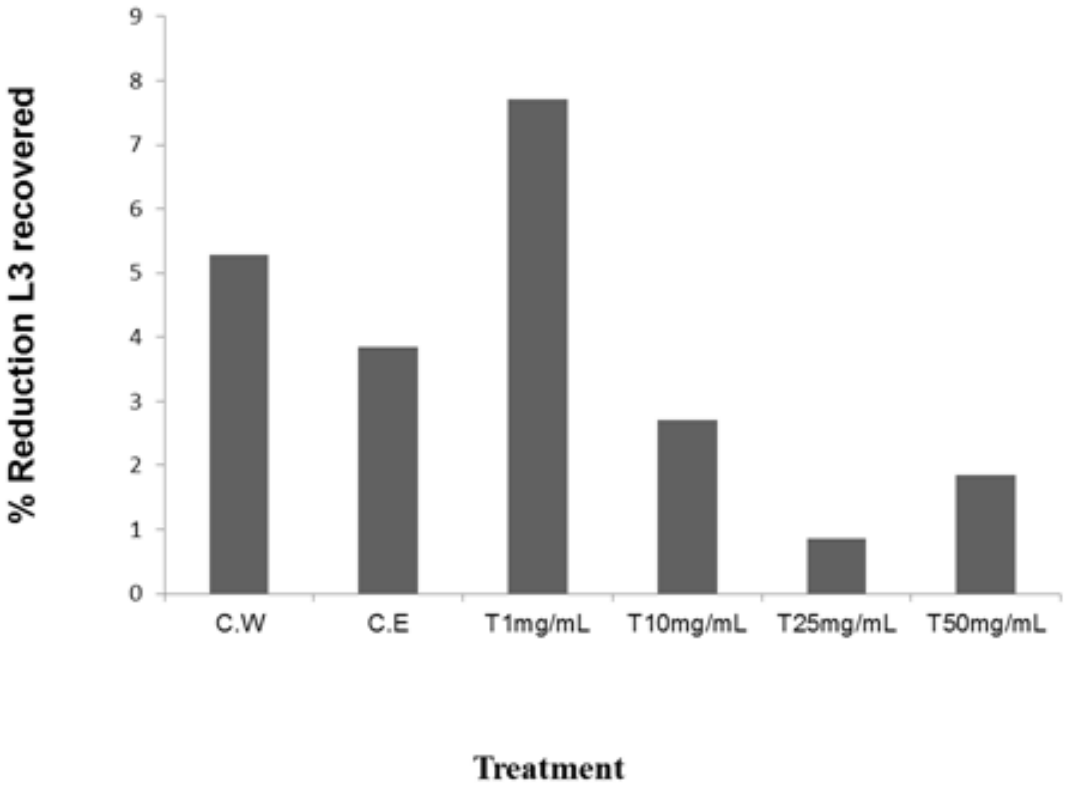

Figure 1. Averages of *Ancylostoma caninum* infective larvae (L3) recovered after treatment with the ethanolic extract of *Mikania glomerata* (0.1 mg/ml, 10 mg/ml, 25 mg/ml, and 50 mg/ml), and the negative water control group and ethanol control after 7 days of interaction. C.W (water control); C.E (ethanol control), T (treatment). ($P < 0.01$) - Tukey test.

instead of aqueous extracts at the same concentration. In that study it was recorded that the methanol extracts showed good anthelmintic activity *in vitro* and *in vivo* and this may be due to the presence of a higher concentration of an alcohol soluble active molecule in the extract (Lone et al., 2012).

Of all the plants that have been studied, the anthelmintic activity was confirmed by *in vitro* or *in vivo* studies and, depending on the plant species or investigated parasite, this activity was or was not confirmed (Sousa et al., 2013; Camurça-Vasconcelos et al., 2005). It is therefore necessary when evaluating the anthelmintic activity of plant extracts to consider at least important factors, including: type of extract, plant part used, concentration / dose, route of administration, bioassay used, infected animal species and parasite species. These factors can interfere with the test and promote a false negative. Therefore, positive results from *in vitro* tests, alone, as well as performed in the present study are not enough to validate researched activity (Camurça-Vasconcelos et al., 2005).

The results showed that the ethanol extract of *M. glomerata* SPRENGEL at different concentrations (1, 10, 25 and 50 mg/ml), exhibited larvicidal activity against gastrointestinal nematode *A. caninum*, the causative agent of CLM, therefore helping families with low conditions, and decreasing treatment costs. Further studies are needed for *in vivo* assays, to improve the methodology and for further clarification of the agents responsible for the observed effects. The use of *in vitro* assays for anthelmintic research from herbal extracts has several advantages, such as ease of implementation, low cost and speed, also serving as an early indication of the activity being investigated and allowing to select the most promising extracts, reducing costs, avoiding loss of time and the indiscriminate use of mice (Stepek et al., 2006; Camurça-Vasconcelos et al., 2005). Thus, this is the first report of *M. glomerata* activity on L3 of *A. caninum*, which no doubt can lead to larger studies to combat other zoonotic geohelminths.

Conflict of Interests

The authors have not declared any conflict of interests.

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