AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

In vitro evaluation of the Anthelmintic Activity of PROMAX-C and propolis extract on the Nematode Parasite *Onchocerca ochengi* Bwangamoi, 1969 (Spirurida: Onchocercidae)

Djongra Mathieu[1*], Mando Ndou Bertrand[1], Djafsia Boursou[1], Tchuenguem Fohouo Fernand-Nestor[1], Ndjonka Dieudonné[1]

1. Department of Biological Sciences, Faculty of Science, University of Ngaoundere, P.O. Box 454 Ngaoundere, Cameroon

ABSTRACT

The anthelmintic activity of the ethanolic and aqueous extracts of Foumban propolis and PROMAX-C was evaluated *in vitro*, on the nematode parasite of cattle *Onchocerca ochengi*. The objective of this study was to find an alternative of synthetic anthelmintic to fight human and animal onchocerciasis. Increasing concentrations of ethanolic and aqueous extracts of propolis and PROMAX-C were prepared in RPMI 1640 culture medium for *O. ochengi* incubation. Dimethylsulfoxide of 0.5 % concentration was the negative control and the positive control was ivermectin. The anthelmintic activity was assessed every 24 and 48 hours and expressed as mortality rate. Acute oral toxicity was assessed in *Mus musculus* for 14 days and subacute toxicity was assessed in males and females of *Ratus norvegicus* for 28 days. Quantification of polyphenols, tannins and flavonoids was performed with a spectrophotometer using gallic acid and rutin as standards. The LC_{50} value was 52.50 ± 0.04 µg/mL for PROMAX-C, while those of the ethanolic and aqueous extracts were 75.50 ± 0.92 and 261.44 ± 18.98 µg/mL, respectively after 48 hours. The test with ivermectin showed a high efficacy on *O. ochengi* nematodes with a LC_{50} value of 100.05 ± 0.35 µg/mL. The ethanolic extract of propolis did not show signs of toxicity on mice. During subacute toxicity, the relative organ weights (kidney, liver, lung, spleen and heart) taken from the rats at the end of treatment did not change significantly. Biochemical showed a decrease in AST and ALT in both sexes at all doses. Thus, the consumption of propolis and PROMAX-C is recommended to fight against onchocerciasis.

Keywords: *Onchocerca ochengi*, anthelmintic, Foumban propolis, PROMAX-C, ivermectin.

*Corresponding Author Email: profndjonka_dede@yahoo.com
Received 10 April 2022, Accepted 20 May 2022

Please cite this article as: Mathieu D *et al.*, In vitro evaluation of the Anthelmintic Activity of PROMAX-C and propolis extract on the Nematode Parasite Onchocerca ochengi Bwangamoi, 1969 (Spirurida: Onchocercidae). American Journal of PharmTech Research 2022.

INTRODUCTION

Onchocerciasis is a parasitosis caused by *Onchocerca volvulus* Leuckart (1893)[1], a filarioidea nematode of the family Onchocercidae and genus *Onchocerca* (Adjami, 2006)[2]. This disease is responsible for more or less chronic skin changes and eye lesions (Jolodar and Miller, 2002)[3]. According to the WHO (2011)[4], more than 102 million people are at risk and, of the 37 million patients worldwide, 99 % of the cases are distributed in 30 countries in intertropical Africa, and the rest in Yemen and parts of Latin America. Onchocerciasis remains a public health problem in developing countries and causes 40,000 cases of blindness each year (WHO, 2011)[4]. In Cameroon, this disease is endemic in the ten regions with variable burden, and its distribution is complex (WHO, 2010)[5]. Six million people are affected and, in the Adamaoua region for example, the prevalence of human and bovine onchocerciasis is estimated around 30 % and 65 %, respectively (WHO, 2010)[5].

From 1987, methods of struggle were set up to control this disease (WHO, 2010)[5]: vector control with spraying of insecticides, nodulectomy and mainly mass treatment with ivermectin (Wahl *et al*., 1998)[6]. However, the expected results of the controls were not reached (Wahl *et al*., 1998)[6], as vector control is weakened by the reduced susceptibility of the fly to insecticides, by its migration over long distances and the reinvasion of previously treated areas (Davies, 1994)[7]. The filaricidal molecules (*e.g*. ivermectin) used so far in the treatment of the disease are only microfilaricidal and thus reduce the transmission of the disease without stopping it (Renz *et al*., 1995)[8]. Moreover, nematode resistance to these products is a serious concern (Lustigman & McCarter, 2007)[9]. Hence the urgent need of developing alternative means to fight river blindness. The exploration of mineral, animal and plant material is therefore a promising avenue. In many parts of the world, the activity of honey bees is being used judiciously in medicine because it is well known (Klein *et al*., 2007)[10], particularly through hive products such as honey, wax, venom and propolis (Liu *et al*., 2013)[11]. Propolis is a resinous substance produced by bees using resin from the buds and bark of certain trees and shrubs (Bankova, 2005)[12]. It has been intensively studied in Eastern European countries and has been used extensively in traditional medicine for the treatment of various ailments (Bankova & Marcucci, 2000)[13]. In cosmetics, propolis is used to effectively solve skin problems: pimples, pustules and acne (Bueno-Costa *et al*., 2016)[14]. It is also used in the treatment of ulcers, cancer, tooth decay and breathing difficulties (Bueno-Costa *et al*., 2016)[14]. PROMAX-C, made by Professor Tchuenguem within the framework of the Association AFH "Abeille-Fleur et Homme" (Declaration No. 309/RDA/H52/BAPP of 17/11/1998 in Ngaoundere, Cameroon), is an ethanolic extract of propolis from the hives of Cameroonian honey bees. It is used in the treatment

of several diseases such as in cough, dental infections, sore throat, angina, wounds, haemorrhoids, gastric ulcers, scabies, ringworm, sinusitis and asthma. Bovine onchocerciasis, caused by *Onchocerca ochengi* (Bwangamoi, 1969)[15], is transmitted by the Diptera *Simulium damnosum* (Wahl *et al*., 1994)[16], a vector of human onchocerciasis. This argument coupled with the phylogenetic relationship between *O. ochengi* and *O. volvulus* supports the use of the cattle parasite as a model for the study of human onchocerciasis (Achukwi *et al*., 2000)[17]. Thus, the general aim of this work is to evaluate the anti-onchocercal effect of natural products (propolis) as a basis for an alternative control of human and bovine onchocerciasis.

MATERIALS AND METHOD

Material

Biological material

The adults of *O. ochengi* were recovered from cow udder nodules harvested at the communal slaughterhouse of Ngaoundere 2[e] (Cameroon). *Mus musculus* Swiss mice, aged of 8 - 9 weeks and weighing 20 - 25 g were used for the acute toxicity study and *Ratus norvegicus*, aged of 8 - 9 weeks and weighing 78 - 95 g were used for the sub-acute toxicity study. These animals were supplied by the National Veterinary Laboratory (LANAVET) of Garoua (Cameroon). We sampled 1 kg of propolis collected in May 2018 in the Noun Division (Western Region of Cameroon).

Chemical material

All chemical were purchased from Sigma (Deisenhofen, Germany) and PROMAX-C (500 mL) was supplied by Professor Tchuenguem (University of Ngaoundere, Cameroon).

Methods

Preparation of the ethanolic and aqueous extracts of propolis

The ethanolic and aqueous extracts of propolis were carried out as described by Mbawala *et al*. (2009)[18]. In practice, 100 g of propolis previously dried at 25 °C and crumbled by hand were macerated for 48 hours in 240 mL of 70 % (v/v) ethanol and for 72 hours in distilled water. The ethanol and aqueous suspension were then separated by centrifugation at 1000 rpm for 10 minutes, and the supernatant was collected and filtered through a filter paper (Whatmann No 1). The filtrate solutions were concentrated under vacuum rotavapor (BUCHU) at 40 °C. The concentrated solution obtained was steamed by an incubator at 40 °C and the resulting extract was weighed, placed in a flask and kept at + 4 °C in a refrigerator. Thus, the extraction yield (in percentage) was determined by the following formula: r = (m/M) 100, where r the extraction yield (%), m the mass of the extract obtained and M the mass of the initial powder.

Preparation of the stock solution of the extract and determination of the different concentrations

The stock solution of the ethanolic extracts of propolis was prepared according to the protocol described by Ndjonka *et al*. (2011)[19]. Briefly, 0.1 g of extract was dissolved into 50 µL of dimethylsulphoxide (DMSO, 100 %), mixed by vortexing and 950 µL distilled water was added. The stock solution obtained was 100 mg/mL and the final volume was 1000 µL. But, the stock solution of aqueous extract of propolis was prepared without DMSO. From stock solutions, the series of dilutions were made to obtain the intermediate solutions.

For the determination of the concentration of the PROMAX-C stock solution, a volume of 3 mL of the latter was evaporated at 40 °C in a stainless steel container that had been previously weighed. This gave the ratio of 0.17 g/ 3 mL (56.66 mg/mL).

Phytochemical analyses

Phytochemical screening of propolis and PROMAX-C

The screening of phytochemicals was made to search for the presence of the probable active phytochemical compounds in the extracts of propolis and PROMAX-C. For all qualitative tests, 1 mL of each extract was used according to the literature (Ndjonka *et al*., 2011; Fankam *et al*., 2011)[19, 20].

Quantitative determination of secondary metabolites

The phenolic content of each sample was determined by the method described by Boizot & Charpentier (2006)[21]. To 100 µL of the solution to be studied, 500 µL of Folin-Ciocalteu reagent was added, followed by 400 µL of a sodium carbonate solution (Na_2CO_3, 7 % w/v). The final solution was stirred and incubated for 10 minutes in the dark. The absorbance was measured at 760 nm against a blank made of distilled water using a spectrophotometer (Biowave). The phenolic compound content of each sample was calculated from a calibration curve established with gallic acid and expressed in mg gallic acid equivalent per 100 g of dry matter (mg GAE/100 g DM).

The determination of the flavonoid content in the samples was carried out according to the method described by Boizot & Charpentier (2006)[21] modified. Thus, to 1 mL of the sample was added 1 mL of $AlCl_3$ (2 % in methanol). After 30 min of incubation in the dark, the absorbance was measured at 430 nm, using a spectrophotometer. A calibration curve (y = ax + b) performed by rutin under the same operating conditions as the sample allowed the quantification of flavonoids in mg rutin equivalent per 100 g dry matter (mg RE/100 g DM).

The total tannin content was determined by the method of Schanderl (1970)[22]. In fact, to 1 mL of the test solution was added 0.5 mL of Folin-Ciocalteu reagent, followed by 5 mL of sodium

carbonate (35 %, w/v). The mixture was incubated for 5 min at 25 °C and then the absorbance measured at 640 nm against a blank (distilled water). A calibration curve (y = ax + b) was carried out using gallic acid (0 to 0.5 mg/mL) at the same conditions as the sample and the amount of tannin was expressed in milligram equivalent of gallic acid per 100 g of dry matter (mg EAG/100 Ms).

The determination of saponin content was assessed by the method of Kumaran and Karunakaran (2006)[23]. Thus, 0.5 g of sample was introduced into a test tube; 5 mL of distilled water was added. The tube was shaken vigorously for 30 sec. Immediately afterwards (5 to 10 sec), the height of the foam formed was measured with a ruler graduated to the nearest 0.1 cm.

Isolation of adult males of *Onchocerca ochengi*

The parasitic nematode of cattle *O. ochengi* lives encapsulated in skin or subcutaneous nodules in the udder of cows or the bursa of bulls. Thus, portions of skin taken from animals slaughtered at the communal slaughterhouse of Ngaoundere 2[e] were isolated from adult males of this parasite as described by Ndjonka *et al.* (2011)[19]. Briefly, the hide portions were first washed with clean water, then disinfected with 70 % ethanol. Then, nodules were isolated from them using a scalpel by making a small incision on the inner side of the skin. These nodules were then cleaned of excess host tissue and placed in a solution of PBS (Phosphate Buffered Saline). The operation continued with the dissection of these nodules, using a scalpel and thick forceps. In fact, the nodules are grasped between the jaws of the forceps ; a small incision made on the nodular wall followed by a slight pressure of the forceps on the nodule leads to the exit of its contents. Under the binocular magnifying glass, the males, which are distinguished from the females by their small size and therefore easier to handle. They were isolated using a mounted needle and soft forceps and placed in a sterile PBS solution. All material (forceps, scalpel, mounted needle, etc.) was previously autoclaved and disinfected with 70 % ethanol during the handling of the worms.

Survival of *Onchocerca ochengi*

Onchocerca ochengi has an *in vitro* maintenance medium, RPMI 1640 (Gibco) in the laboratory. Thus, 144 worms were incubated at 37 °C, 6 worms per well in 24-well plates, in the presence of 300 µL of a sterile solution of RPMI-1640 (with 25 µg/mL of penicillin and streptomycin) and 0.08 % dimethyl sulphoxide (DMSO). This was done after the worms were washed 3 times in PBS and then twice in RPMI-1640 in a sterile hood. The viability of the worms was determined every 24 hours (after 24 hours for the first row), after 48 hours for the second, and so on until the 16th row (Ndjonka *et al.*, 2011)[19].

Evaluation of the *in vitro* sensitivity of adult males of *Onchocerca ochengi* to ethanolic and aqueous extract of propolis and PROMAX-C.

The effect of each product was evaluated according to the modified method of Ndjonka *et al.* (2011)[19]. Thus, for each concentration, 6 individuals were incubated (1/well) with different concentrations of propolis extracts in RPMI-1640 supplemented with penicillin and streptomycin. Thus, each well was first given 100 µL of RPMI-1640 and then the corresponding volume of extract from the stock solution was added after the same volume of RPMI had been removed. The effects of ethanolic and aqueous propolis extract and PROMAX-C were evaluated isolately. The positive control was performed with ivermectin and the negative one was made of 5 % DMSO. The tests were performed in three independent replicates for 24 and 48 h in a 37 °C incubator.

Determination of mortality of *Onchocerca ochengi* adults

To ensure the viability of the worms after the tests, the methyl tetrazolium colorimetric test (MTT) was performed. In principle, MTT is a pale yellow compound that is reduced to a dark blue product, formazan, by living cells. In practice, the worms previously washed with PBS were incubated 6 per well (worms from the same test concentration) in 500 µL of RPMI-MTT solution (0.5 mg/mL in PBS). The worms did 30 min at 37 °C incubation in the 24-well plates. After incubation, purple (live) and yellow (dead) worms were observed under a binocular magnifying glass. All MTT tests were performed in the dark, as the product is sensitive to light (Loveland *et al*., 1992)[24].

Acute and subacute oral toxicity tests of ethanolic extract of Foumban propolis (EEP) on mice and white rats

Acute toxicity test on white mice

The toxicity test was conducted according to the "dose adjustment" method of OECD line 425 OECD (2008a)[25] consisted of testing the ethanolic extract of propolis at a dose of 2000 mg/kg. The test was performed on 6 female *Mus musculus* mice and their behaviour was observed as well as the number of deaths over a period of 14 days. After 15 h of fasting, they were divided as follows: control batch consisting of 3 females receiving distilled water at a rate of 10 mL/kg; experimental batch consisting of 3 females receiving the extract at a dose of 2000 mg/kg. Behavioural observation was performed 3 h post administration of the substances. Subsequently, hydration and feeding were carried out on a daily basis for 14 days. During this period, the signs of toxicity, in particular the modification of the coat, motility, tremors, grooming, respiration, sensitivity to noise after a metallic shock, the appearance of the faeces, mobility, and death were noted.

Subacute toxicity test in rats

It was determined from OECD Guideline 407 (OECD, 2008b)[26]. 24 albino Wistar rats were randomly distributed in the cages into four equal batches of 3 males and 3 females as follows : batch 1, receiving distilled water at 1 mL/100 mg body weight (control batch); batches 2, 3 and 4 receiving a solution of the ethanolic extract of propolis at 250, 500, 1000 mg/kg body weight respectively. During the 4 weeks of study, clinical signs and toxicity were observed daily in all animals, before and immediately after administration of the ethanolic extract of propolis. The rats were fed and hydrated *ad libitum* and weighed every 2 days. At the end of the treatment, the rats were fasted for 24 h, blood was taken for biological analysis, and then, after sacrifice, the livers, kidneys, hearts, rats and lungs were removed and rinsed with 0.9 % saline solution, weighed and preserved in 10 % formalin for histopathological examination.

Data analysis

LC_{50} values were calculated using Log-probit method with SPSS 16.0 software. Data were expressed as mean ± standard error on the mean (M ± SEM). Data comparison was done using analysis of variances (one way-ANOVA) followed by multiple tests of comparison of Bonferroni. The curves were plotted using Graph Pad prism 5.10. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Ethanolic and aqueous extraction yield of propolis

The ethanolic and aqueous extracts obtained by maceration of propolis were brown in color. The yields were 11.66 % for the ethanolic extract and 4.7 % for the aqueous extract. The evaluation of the extraction yield of propolis shows that the ethanolic extract exhibited a higher yield compared to the aqueous extract. This difference in yield observed between aqueous and ethanolic extract is due to the fact that ethanol extracts contains more phytochemicals than water extract. Our results are higher than that reporting by Talla *et al*. (2013)[27]. These authors obtained yield of 5.5 % on Ngaoundal propolis, with ethanol extraction. The differences in the extraction a yield could be related to the places where the propolis is harvested. The differences in extraction yield and concentrations are linked to the characteristics of crude propolis influenced by the harvest season, and regional flora (Popova *et al*., 2010b)[28].

Phytochemical composition of propolis and PROMAX-C

The evidence of several chemical groups was made, namely polyphenols, flavonoids, tannins, alkaloids, terpenes, steroids and saponins.

The qualitative phytochemical study of the ethanolic extract of propolis shows the presence of saponins, tannins, flavonoids, terpenoids, polyphenols and alkaloids. The phytochemical

characterization of PROMAX-C revealed only the presence of polyphenols, tannins, saponins and flavonoids (table 1).

Table 1: Summary table of the results of the phytochemical screening of propolis and PROMAX-C

Secondary metabolites	Ethanolic extract	Aqueous extract	PROMAX-C
Saponines	+	+	+
Alcaloids	+	+	-
Tannins	+	+	+
Polyphenols	+	+	+
Flavonoids	+	+	+
Terpenoids	+	-	-

+ : present ; - : absent

Recent studies by Popova *et al*. (2010b)[28] revealed that the composition of propolis varied greatly from one sample to another. According to these authors, this variation is not only qualitative, but also quantitative. On the whole, the results obtained by Mbawala *et al*. (2009)[18] and Talla *et al*. (2013)[27], are similar to ours, as the same compounds were also found in the propolis samples. Njintang *et al*. (2012)[29] reported the presence of triterpenoids in PROMAX-C. This result is different from ours. These differences could be related to the harvesting periods and of the botanical origin of propolis (Koru, 2007)[30].

Quantitative composition of some secondary metabolites in propolis and PROMAX-C

Total phenolic compounds, tannins, flavonoids and saponins were quantified using a spectrophotometer. The amount of each secondary metabolite varies greatly from one extract to another (table 2).

Table 2: Quantitative phytochemical composition of propolis and PROMAX-C

Extracts	Compounds			
	Polyphenols (mg GAE/100 g)	Flavonoids (mg RE/100 g)	Tannins (mg GAE/100 g)	Saponins (mg/g)
Ethanolic	274.70 ± 0.05	139.40 ± 0.03	171.49 ± 0.04	28.80 ± 0.30
Aqueous	80.50 ± 0.19	39.29 ± 0.24	50.14 ± 0.66	19.40 ± 0.20
PROMAX-C	100.44 ± 0.02	38.80 ± 0.01	62.51 ± 0.02	14.40 ± 0.15

GAE: Gallic acid equivalent; RE: Rutin equivalent. Values are expressed as mg Gallic acid or rutin equivalent per 100 g dry matter.

Sensitivity of male *Onchocerca ochengi* to PROMAX-C sample, ethanolic and aqueous extract of Foumban propolis and ivermectin

Effect of ethanolic and aqueous extract of Foumban propolis on *Onchocerca ochengi*

Figure 1 illustrates the variations in the mortality rate of adult males *Onchocerca ochengi* after 24 h and 48 h of incubation as a function of the concentrations of the ethanolic and aqueous extracts of propolis.

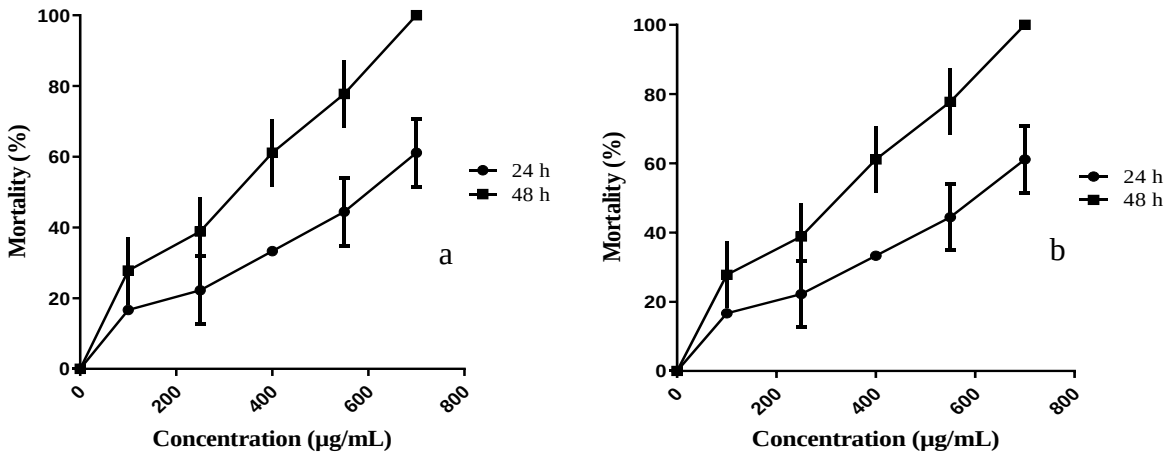

Figure 1: Mortality rate of *Onchocerca ochengi* as a function of concentrations of ethanolic extract (a) and aqueous extract (b) of Foumban propolis.

Phenolic compounds are endowed with multiple biological effects, including antioxidant activity (Ahn *et al.*, 2007)[31]. Propolis affects the cytoplasmic membrane, inhibits bacterial motility and enzymatic activity, exhibits bacterial activity against different bacterial genera and may be bactericidal in high concentrations (Mirzoeva *et al.*, 1997)[32].

Effect of PROMAX-C and ivermectin on adult males of *Onchocerca ochengi*

The effect of PROMAX-C on *O. ochengi* is concentration dependent, with a mortality rate of 100 % achieved at a concentration of 130 µg/mL after 48 hours (figure 3a). The average value of LC_{50} of 3 repetitions of tests of the sample of the PROMAX-C performed on *O. ochengi* is 52.50 ± 0.04 µg/mL l after 48 hours of incubation at 37 °C. Figure 3b show the mortality rate of *O. ochengi* as a function of ivermectin concentration. The mean LC_{50} value of 3 repetitions of ivermectin tests carried out on *O. ochengi* is 100.05 ± 0.35 µg/mL after 48 hours of incubation at 37 °C.

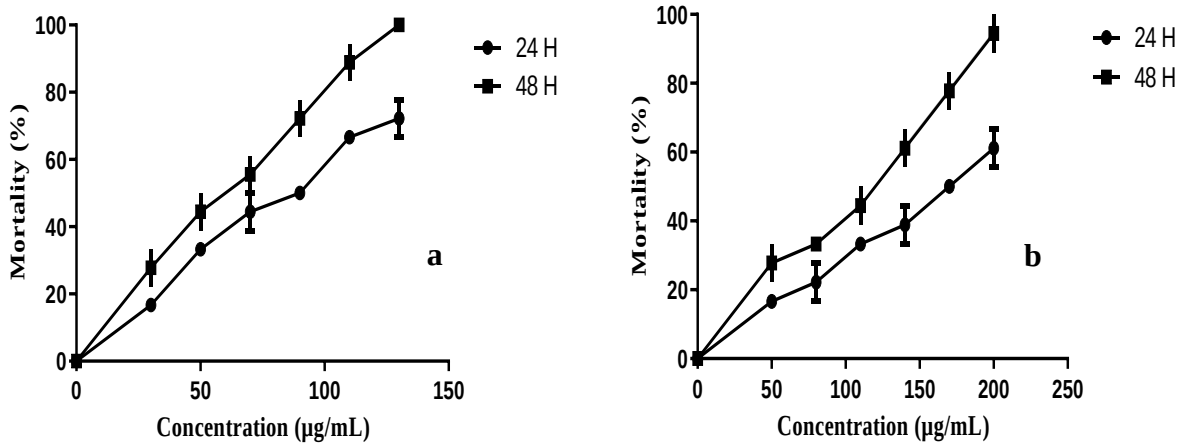

Figure 2: Mortality rate of *Onchocerca ochengi* as a function of PROMAX-C (a) and ivermectin (b) concentrations.

The nematode mortality could be explained by the components of propolis that are active against nematodes (Ahn *et al*., 2007)[31]. The mechanisms of action of the reference drug (ivermectin) are known. The most common effect of dewormers against infections is paralysis of the parasite's muscles or inhibition of neuromuscular transmission or enzymes involved in energy production. Other drugs damage the integument and allow digestion or partial rejection by the host immune system (Mirzoeva *et al*., 1997)[32].

Comparison of the effects of lethal concentrations of ethanolic, aqueous propolis extract, PROMAX-C and ivermectin performed on adult males of *Onchocerca ochengi*

Table 3 shows the mean values of LC_{50} of the extracts of propolis, the sample of PROMAX-C and ivermectin.

Table 3: Lethal concentrations (LC_{50} in mg/mL) of ethanolic, aqueous propolis extract, PROMAX-C and ivermectin on the parasite *Onchocerca ochengi* obtained in 24 h and 48 h.

Extracts	LC_{50} ± SD (µg/mL) 24 h	LC_{50} ± SD (µg/mL) 48 h
Ethanolic	166.69 ± 0.65	75.50 ± 0.92*
Aqueous	580.79 ± 35.50***	261.44 ± 18.98**
PROMAX-C	80.70 ± 0.05**	52.50 ± 0.04*
Ivermectin	175.56 ± 0.75	100.05 ± 0.35

* $P > 0,05$; ** $P < 0,01$ and *** $P < 0,001$.

The comparison of LC_{50} values reveals no difference between the ethanolic extract of propolis and ivermectin ($P > 0,05$). However, the comparison of the LC_{50} values reveals a very significant difference between the PROMAX-C sample and the ivermectin ($P < 0.01$). This result suggests that the ethanolic extract of propolis and the sample of PROMAX-C as well as ivermectin would act in the same way. The mortality of adult males of *Onchocerca ochengi* could be due to the

phytochemicals present in the ethanolic extract of propolis and the sample of PROMAX-C. Ivermectin has an action on two chloride ion transporters, one of which is a glutamate receptor present in nematodes and the other is the GABA receptor (gamma-amino butyric acid). They are agonists of the gamma-amino butyric acid receptor and the glutamate receptors. They cause flaccid paralysis of the parasite by increasing membrane permeability to chloride ions (Martin, 1997)[33]. At low concentration, its binding to the receptors potentiates the effect of the neurotransmitter, it irreversibly opens the channel dependent on the receptor. This opening causes a current of chloride ions which induces axonal hyperpolarization and flaccid paralysis (Ahoussou, 2007)[34].

The tannins according to Massamha *et al*. (2010)[35] would react on directly with the surface proteins of the parasite *O. ochengi*, this resulting in a physiological dysfunction in the nematodes such as the mobility and the absorption of food, what would cause the worm to die. Prashant *et al*. (2011)[36] highlighted in their work the activity of tannins, polyphenols, flavonoids and saponins on nematodes. Fatty acids interfere with many stages of inflammation, such as contraction vascular, chemotaxis, cell adhesion and cell activation (Looi *et al*., 2013)[37]. The modulate leukocyte function, control proliferation, production of cytokines and adhesion molecules and cause cell death (Looi *et al*., 2013 ; Arghiani *et al*., 2014) [37, 38].

Acute toxicity

During the 14 days of observation after gavage, no death mice were observed in the experiment, which continued to lead an apparently normal life. No signs of toxicity such as decreased sensitivity to noise or locomotion were observed. The general behavior of the mice remains normal in comparison with the controls. The propolis extract administered orally at a dose of 2000 mg/kg did not cause any deaths throughout the study: the extract has a toxicity index equivalent to 5 (which is non-toxic), according to the toxicity scale for a chemical based on the LD_{50} and the route of administration (Looi, *et al*., 2013)[37]. No signs of toxicity were observed for 4 h after administration of the extract.

Subacute toxicity

Effect of the ethanolic extract of propolis on the body weight of animals

After 28 days of treatment, no mortality was observed during the experiment. It can be seen from the results that the control batch containing the rats force-fed with distilled water and the batches treated with the ethanolic extract of propolis during the experiment. No differences were observed in the weight changes of either male or female rats ($P < 0.05$). The effects related to the daily oral administration in repeated doses of the ethanolic extract of propolis were appreciated after the

evaluation of the behavioral parameters, weight growth, relative organ weight and biochemical parameters. However, there was no significant difference in mass at the last weighing (day 28).

Effect of the ethanolic extract of propolis on the weight of the organs

The data of table 4 from the organs weight did not apparently present any difference following the administration of ethanolic extract and propolis doses.

Table 4: Effects of ethanolic extract of propolis on relative organ weights in a dose-dependent manner in males and females.

Parameters	Doses (mg/kg)				
		Control	Batch 250	Batch 500	Batch 1000
Liver	M	0.40 ± 0.01	0.44 ± 0.01	0.38 ± 0.01	0.39 ± 0.01
	F	0.39 ± 0.02	0.41 ± 0.01	0.41 ± 0.04	0.38 ± 0.01
Heart	M	0.80 ± 0.04	0.82 ± 0.18	0.80 ± 0.11	0.87 ± 0.09
	F	0.94 ± 0.02	0.84 ± 0.06	0.97 ± 0.13	0.86 ± 0.01
Kidneys	M	0.91 ± 0.05	1.00 ± 0.12	0.80 ± 0.08	0.85 ± 0.05
	F	1.04 ± 0.12	0.99 ± 0.05	0.95 ± 0.03	0.96 ± 0.06
Lung	M	4.12 ± 0.28	4.69 ± 0.66	4.30 ± 0.16	4.20 ± 0.15
	F	4.96 ± 0.16	4.77 ± 0.42	4.24 ± 0.11	4.20 ± 0.23
Spleen	M	0.25 ± 0.02	0.27 ± 0.02	0.25 ± 0.03	0.26 ± 0.05
	F	0.25 ± 0.09	0.27 ± 0.03	0.26 ± 0.05	0.24 ± 0.02

Values are means ± standard errors, with each batch comprising 6 animals. M: male and F: female.

Concerning the relative weights of the organs (kidneys, liver) overall, no statistically significant differences were found between the control and the different doses of the extract for these parameters.

Effect of the ethanolic extract of propolis on biochemical parameters

The ethanolic extract of propolis seems not to induce any significant change in the concentration of urea creatinine and albumin after 28 days (Table 5).

Table 5: Effect of ethanolic extract of propolis on biochemical parameters of male and female rats after 28 days of oral treatment.

Parameters	Doses (mg/kg)				
		Control	Batch 250	Batch 500	Batch 1000
ASAT (IU/L)	M	107.00 ± 12.02	91 ± 13.49	77.33 ± 8.37*	73.66 ± 18.34*
	F	105.66 ± 14.61	73.66 ± 9.74*	68.50 ± 15.50**	59.20 ± 14.16**
ALAT (IU/L)	M	100.66 ± 11.11	84 ± 13.95*	71.33 ± 10.65*	68 ± 19.79**
	F	101.23 ± 18.23	80.33 ± 9.93*	74.50 ± 6.50*	65.35 ± 12.56**
Urea (mg/L)	M	31.03 ± 3.01	28.86 ± 1.76	29.26 ± 10.21	32.93 ± 16.31
	F	28.80 ± 8.17	27.23 ± 14.07	26.95 ± 2.05	27.46 ± 10.98
Creatinine	M	0.63 ± 0.04	0.66 ± 0.04	0.42 ± 0.28*	0.53 ± 0.63
(mg/L)	F	0.58 ± 0.18	0.63 ± 0.54	0.60 ± 0.10	0.63 ± 0.24
Albumin	M	41.50 ± 2.72	42.26 ± 4.23	39.36 ± 2.44	42.56 ± 1.54
	F	40.80 ± 3.88	40.93 ± 2.33	41.10 ± 1.50	40.90 ± 0.56

Values are means standard errors, with each batch comprising 6 animals. *$P < 0.05$; **$P < 0.01$. M: male; F: female.

The analyses performed showed a decrease in ASAT and ALAT in both sexes at all doses. ALAT and ASAT levels rise rapidly when the liver is damaged for a variety of reasons including hepatic cell necrosis, hepatitis, cirrhosis and hepatotoxicity of certain drugs (Arghiani *et al*., 2014)[38]. The concentration of ALAT and ASAT was significantly decreased in animals treated with all the doses (250, 500 and 1000 mg/kg) in our study. Significant reductions in ASAT levels have already been obtained by Veerakumari & Priya (2006)[39], with the aqueous extract of *Artemisia afra* leaves in rats. Our results show that EEPF could have a hepatoprotective effect in these animals. Moreover, this hypothesis seems to be confirmed by the work of Ramasamy *et al*. (2009)[40]. These authors demonstrated that the ethanolic extract of the fruits of *Passiflora foetida* exerts a hepatoprotective effect in the case of hepatotoxicity induced by carbon tetrachloride (CCl4).

Effect of the ethanolic extract of propolis on hematological parameters

The ethanolic extract of propolis on the hematological parameters showed a decrease in the mean corpuscular concentration of hemoglobin at a dose of 250 mg/kg of body weight compared to the control batch for female rats. There is also a decrease in red blood cells at a dose of 1000 mg/kg of body weight compared to the control batch for female rats (Table 6).

Table 6: Effect of ethanolic extract of propolis on hematological parameters of female and of male rats after 28 days of oral treatment.

Parameters	Doses (mg/kg)				
		Control	Batch 250	Batch 500	Batch 1000
White blood cells ($\times 10^3$ µL)	M	12.03 ± 2.59	19.10 ± 14.10	7.45 ± 2.95	8.80 ± 2.85
	F	8.80 ± 3.32	8.26 ± 2.19	9.40 ± 3.39	8.96 ± 2.15
Lymphocytes (%)	M	77.40 ± 5.48	74.43 ± 11.89	73.05 ± 1.35	78.76 ± 3.29
	F	86.36 ± 2.73	84.23 ± 1.89	83.23 ± 2.45	82.4 ± 11.90
Red cells ($\times 10^6$ µL)	M	8.33 ± 0.20	7.36 ± 0.85	6.86 ± 3.10	5.83 ± 3.24*
	F	6.05 ± 2.15	5.79 ± 3.04	5.36 ± 1.76	4.69 ± 0.29*
Hemoglobins (g /dL)	M	15.36 ± 0.24	14.40 ± 3.75	15.70 ± 0.40	14.20 ± 2.74
	F	14.93 ± 3.92	13.90 ± 1.09	15.53 ± 0.77	14.93 ± 0.85
Hematocrits (%)	M	42.86 ± 1.55	39.13 ± 9.22	38.20 ± 16.00	41.33 ± 7.30
	F	39.86 ± 13.92	40.96 ± 6.41	40.23 ± 4.98	42.56 ± 1.48
Platelets	M	562.66 ± 132.38	523.33 ± 125.35	349.5 ± 69.50*	491.33 ± 227.21
	F	402.66 ± 243.31	465.66 ± 167.85	457.66 ± 67.60	489.33 ± 122.25

Values are means ± standard errors, with each batch comprising 6 animals. *$P < 0.05$ compared with the control on the same line. M: male and F: female.

In this study, administration of the ethanolic extract of propolis to animals (males) resulted in a non-significant reduction in blood creatinine levels at a dose of 500 mg/ kg. According to Rock *et al*. (1987)[41], a reduction in blood creatinine suggests a non-lethal effect of the extracts on the

kidneys and the increase in creatinine is due to renal dysfunction particularly a reduction in glomerular filtration rate.

Histopathological examination

Figure 3 show histological sections of the liver of female rats. Histopathological study of the liver did not reveal any case of inflammation in the animals treated with the different doses of propolis extract.

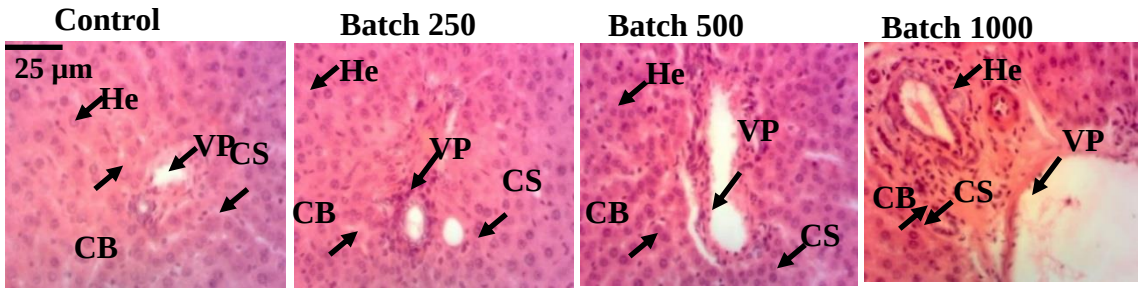

Figure 3: Microphotographs of the histological section of the liver (X200) of female rats treated with different doses. CB: bile canaliculi, VP: hepatic portal vein, CS: sinusoidal capillary, He: hepatocytes.

Figure 4 show histological sections of the kidney of female rats. Histopathological study of the kidney did not reveal any case of hepatic cell necrosis in the animals treated with the different doses of propolis extract.

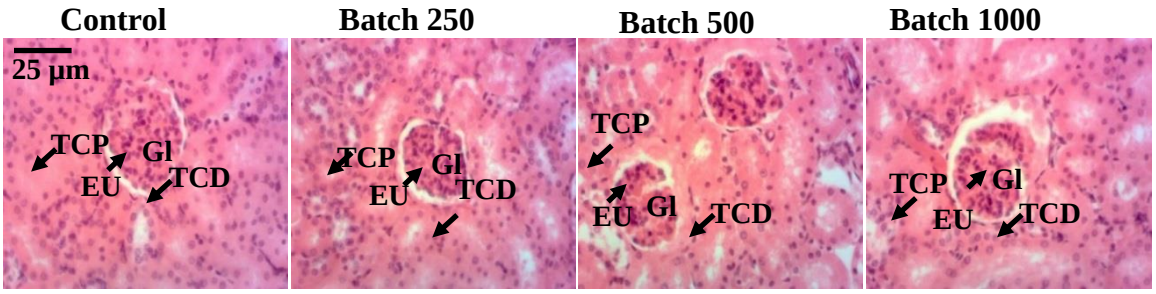

Figure 4: Microphotographs of the histological section of the kidney (X200) of female rats treated with the different doses. GL: glomeruli, TCP: proximal involved tube, TCD: distal involved tubule, EU: urinary space.

Figure 5 show histological section of the liver of male rats. Histopathological study of the liver did not reveal any cases of inflammation in the animals treated with the different doses of propolis extract.

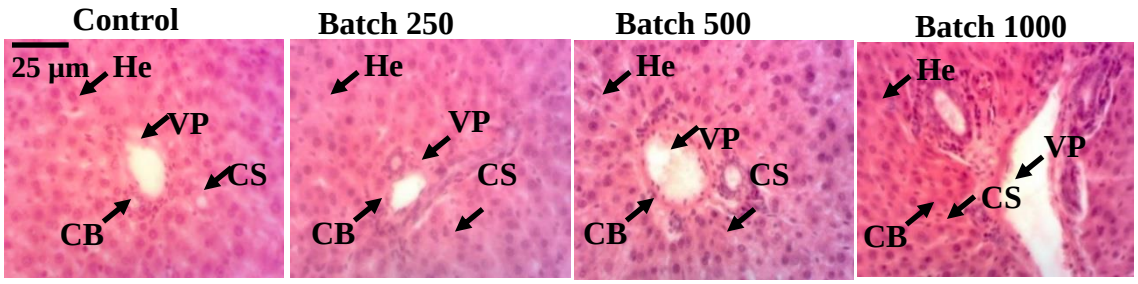

Figure 5: Microphotographs of the histological section of the liver (X200) of male rats treated with the different doses. Cb: Bile Canaliculi, VP: Hepatic Portal Vein, CS: Sinusoidal Capillary, He: Hepatocytes.

Figure 6 show histological section of the kidney of male rats. Histopathological study of the kidney did not reveal any cases of hepatic cell necrosis in the animals treated with the different doses of propolis extract.

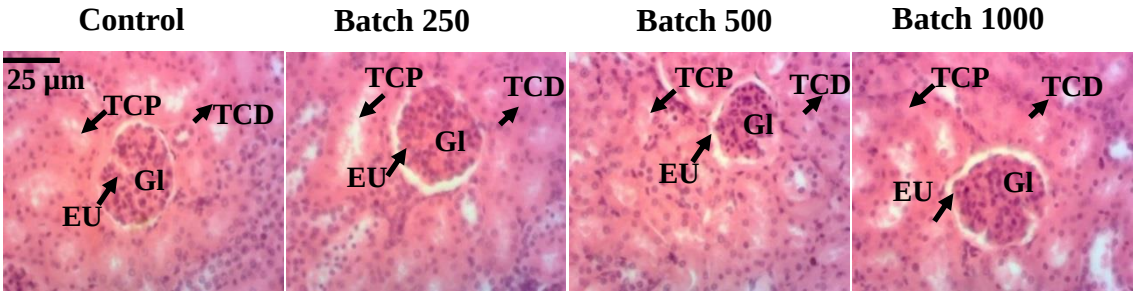

Figure 6: Microphotographs of the histological section of the kidney (X200) of male rats treated with the different doses. GL: glomeruli, TCP: proximal involved tube, TCD: distal involved tubule, EU: urinary space.

No histopathological sign, was observed in the different batches involved in the present investigation, irrespective of sex or organ considered. This result seems to confirm the data from the biochemical analysis concerning a possible hepatoprotective action of the ethanolic extract of propolis.

CONCLUSION

The evaluation of the activity of PROMAX-C, ethanolic and aqueous extract of Foumban propolis on *O. ochengi* revealed that all three extracts tested have a remarkable lethal effect on *O. ochengi*. The mortality rate of the worms was dose-dependent of each extract. PROMAX-C was more nematotoxic to male *O. ochengi* worms than the ethanolic and aqueous extract of Foumban propolis (Cameroon). Overall, PROMAX-C, the ethanolic and aqueous extract of Foumban propolis, is a cocktail of bioactive molecules that could be responsible for the observed nematicidal

activity. The presence of secondary metabolites in our extracts is the source of their anthelmintic activity on the nematode *O. ochengi*.

REFERENCES

1. Leuckart WGF. Diseases of the skin in tropical climates. Trans. 7th Int. Congres Hygiene Journal 1893; 1-93.
2. Adjami AGA. Contribution à la surveillance entomologique et épidémiologique de l'onchocercose en Afrique de l'Ouest. Thèse de Doctorat en Parasitologie, Université de Montpellier II, Sciences et Techniques du Languedoc, France 2006; 234 p.
3. Jolodar A. & Miller DJ. Construction and expression of aspartic protease from *Onchocerca volvulus* as ompA fusion protein in a mutant strain of Salmonella typhimurium. Genetics and Molecular Biology 2002; 25: 97-101.
4. WHO. Taking action to reduce the global impact of neglected tropical diseases: the first WHO. Report on neglected tropical diseases 2011; 169 p.
5. WHO. African Onchocerciasis Control Programme 2010; 28-80 p.
6. Wahl G, Enyong P, Ngosso A, Schibel JM, Moyou R, Tubbesing H, Ekale D & Renz A. *Onchocerca ochengi*: epidemiological evidence of cross-protection against Onchocerca volvulus in man. Parasitology 1998; 116: 349-362.
7. Davies JB. Sixty years of onchocercciasis vector control: a chronological summary with comments on eradication, reinvasion and insecticide resistance. Annual Review Entomology 1994; 39: 23-45.
8. Renz A, Trees AJ, Achu-kwi MD, Edwards G & Wahl G. Evaluation of Suramin, Ivermectin and CGP 20376 in a new macrofilaricidal drug screen, *Onchocerca ochengi* in Africa cattle. Tropical Medicine and Parasitology 1995; 46: 31-37.
9. Lustigman S & McCarter JP. Ivermectin resistance in *Onchocerca volvulus* : toward a genetic basis. PLoS Neglected Tropical Disease 2007; 1(1): 76.
10. Klein AM, Vaissiere BE, Cane JH, Dewenter IS, Cunningham SA, Kremen C & Tscharntke T. Importance of pollinators in changing landscapes for world crops. Review 2007; 274: 303-313.
11. Liu R, Zhang H, Yuan M, Zhou J, Tu Q, Liu JJ & Wang J. Synthesis and biological evaluation of apigenin derivatives as antibacterial and antiproliferative agents. Molecules 2013; 18: 11496-11511.
12. Bankova V. Recent trends and important developments in propolis research. CAM 2005; 2: 29-32.

13. Bankova V & Marcucci MC. Standardization of propolis: present status and perspectives. Bee World 2000; 81: 182-188.
14. Bueno-Costa FM, Zambiazi RC, Bohmer BW, Chaves FC, Silva WPD, Zanusso JT & Dutra I. Antibacterial and antioxidant activity of honeys from the state of Rio Grande do Sul, Brazil. LWT - Food Science and Technology 2016; 65: 333-340.
15. Bwangamoi O. Dermatitis in cattle caused by *Onchocerca ochengi*, and the effect of the adult filarial on the finished leather. Bulletin Epizootic Disease Africa 1969; 17: 321-335.
16. Wahl G, Achukwi DM, Mbah D, Dawa O & Renz A. Bovine onchocerciasis in North Cameroon. Veterinary Parasitology 1994; 52: 297-311.
17. Achukwi MD, Harnett W & Renz A. *Onchocerca ochengi* transmission dynamics and the correlation of *Onchocerca ochengi* microfilaria density in cattle with the transmission potential. Veterinary Research 2000; 31: 611-621.
18. Mbawala A, Tchuenguem FF-N, Roger D & Millière JB. Spectra of antibacteria activity of propolis (PROMAX-C) samples from two localities of Adamaoua province (Cameroon). Research Journal of Microbiology 2009; 4: 150-157.
19. Ndjonka D, Agyare C, Lüersen K, Djafsia B, Achuki D, Nukenine EN, Hensel A & Liebau E. In vitro activity of Cameroonian and Ghanaian medicinal plants on parasitic (*Onchocerca ochengi*) and free-living (*Caenorhabditis elegans*) nematodes. Journal Helminthology 2011; 85: 304-312.
20. Fankam AG, Kuete V, Voukeng IK, Kuiate RJ. & Pages J-M. Antibacterial activities of selected Cameroonian spices and their synergistic effects with antibiotics against multidrug-resistant phenotypes. Complementary and Alternative Medicine 2011; 11: 104-109.
21. Boizot N & Charpentier JP. Rapid method for assessing the phenolic content of forest tree organs. Le Cahier des Techniques de l'Inra 2006; 79-82.
22. Schanderl SH. Methods in food analysis. Academic Press, New York, London 1970; 709 p.
23. Kumaran A & Karunakaran RJ. Antioxidant and free radical scavenging activity of anaqueous extract of Coleus aromaticus. Food chemistry 2006; 97: 109-114.
24. Loveland BE, Johns TG, Mackay IR, Vaillant F, Wang & ZX Hertzog PJ. Validation of the MTT dye assay for enumeration of cells in proliferative and antiproliferative assays. Biochemestry International 1992; 27: 501-510.
25. OECD. European Pharmacopoeia. 6th edition 2008a; Volume 1. pp. 178-568.
26. OECD. Test Guideline for Chemicals 2008b; 407.

27. Talla E, Dabole B, Taïwe GS, Ngo-Bum E, Mbafor JT, Atchade ADT, Malik R, Zukfiqar A, Sidiki N, Nguimbou RM, & Choudhary MI. Antinociceptive pentacyclic triterpenoids from the Cameroonian brown propolis. Pharmacology 2013; 4: 218-227.
28. Popova M, Chen CN, Chen PY, Huang CY & Bankova VA. Validated spectrophotometric method for quantification of prenylated fla-vanones in Pacific propolis from Taiwan. Phytochemical Analysis 2010b; 21: 186-191.
29. Njintang YN, Tatsadjieu NL, Ngakou A, Danra D & Tchuenguem FF-N. Antiradical activity and polyphenol content of ethanolic extracts of propolis. International Journal of Biological sciences 2012; 2: 56-63.
30. Koru O, Toksoy F, Acikel CH, Tunca YM, Baysallar M, Guclu AU, Akca E, Tuylu AO, Sorkun K, Tanyuksel M & Salih B. In vitro antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens. Anaerobe 2007; 13: 140-145.
31. Ahn MR, Kumazawa S, Usui Y, Nakamura J, Matsuka M, Zhu F & Nakayama T. Antioxidant activity and constituents of propolis collected in various careas of China. Food Chemistry 2007; 101(4): 1383-1392.
32. Mirzoeva OK, Grishanin RN & Colder PC. Antimicrobial action of propolis and some of its components: the effect on growth, membrane potential and motility of bacteria. Microbiological Research 1997; 52: 239-246.
33. Martin RJ, Murray I, Robertson AP, Bjorn H & Sangster N. Anthelmintic and ion-channels: after a puncture, use a patch. International Journal of Parasitology 1998; 28: 849-862.
34. Ahoussou S. Effet du loperamide sur l'efficacité de l'ivermectine et de la moxidectine dans le traitement des strongyloses gastrointestinales chez les bovins en Argentine. Mémoire de thèse. Université Paul Sabatier. Toulouse 2007; 110 p.
35. Massamha B, Gadzirayi CT & Mukutirwa I. Efficacy of Allium sativum (Garlic) controlling nematode parasites in sheep. International Journal and Research of Veterinaire Medicine 2010; 8: 161-169.
36. Prashant T, Bimlesh K, Mandeep K, Gurpreet K & Harleen Kaur. Phytochemical screening and Extraction: A Review. Internationale Pharmaceutica Sciencia 2011; 1: 1-9.
37. Looi CY, Arya A, Cheah FK, Muharram B, Leong KH, Mohamad K, Wong WF, Rai N & Mustafa MR. Induction of apoptosis in human breast cancer cells via caspase pathway by

vernodalin isolated from Centratherum anthelminticum (L.) seeds, PLoS One 2013; 8(2): e56643.

38. Arghiani N, Matin MM, Bahrami AR, Iranshahi M, Sazgarnia A & Rassouli FB. Investigating anticancer properties of the sesquiterpene ferutinin on colon carcinoma cells, in vitro and in vivo, Life Science 2014; 109: 87-94.
39. Veerakumari L. & Priya P. In vitro effect of azadirachtin on the motility and acetylcholinesterase activity of Cotylophoron cotylophorum (Fischoeder, 1901). Journal of Veterinary Parasitology 2006; 20: 1-5.
40. Ramasamy A, Balasundaram J, Sundararaman J, Rajappan M & Raju SK. Effect of fruits of Passiflora foetida Linn. on CCl4 induced hepatic injury in rats. Journal of Pharmatech Researche 2009; 2(3): 413-415.
41. Rock RC, Walker WG & Jenning CD. Nitrogens metabolites and renal function. In: Tietz NW. editors. Fundamentals of Clinical Chemistry. Philadelphia: WB Saunders 1987; 24: 669-704.