Vol. 15(2), pp. 9-15, October-December 2023
DOI: 10.5897/JPP2023.0627
Article Number: 27B83C271320
ISSN 2141-2502

http://www.academicjournals.org/JPP

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Journal of Pharmacognosy and Phytotherapy

Full Length Research Paper

In vitro anthelmintic activity against *Haemonchus contortus* of methanolic extracts of selected medicinal plants from Meru County, Kenya

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Received 7 March, 2023; Accepted 17 May, 2023

Helminthiasis causes losses in livestock production, and anthelminthics are used to treat helmintic infections. Resistance has necessitated the need for development of more effective alternatives. Plants offer a promising alternative and, in Meru County, traditional plant-based treatments for helminthiasis have been used, although there is a lack of scientific proof of their efficacy. The aim of this study was to investigate efficacy of *Bridelia micrantha, Aframomum zambesiacum, Hagenia abyssinica, Rubus apetalus, Thespecia garckeana, Physalis peruviana* and *Caesalpina volkensii* against *Haemonchus contortus*. It entailed screening of methanolic extracts of these plants against *H. contortus*, from sheep farms in Muguga Kenya. Their efficacy was tested *in-vitro* using eggs and larvae of *H. contortus*. Egg hatchability was determined after 48-h incubation with extracts while larvae survival was determined after six days incubation. Physiological saline was the negative control while albendazole was the positive control. One-way ANOVA was conducted followed by Tukey's test. *P. peruviana* and *R. apetalus,* inhibition percentages of 95.24±0.54, 90.00±1.00, 88.24±0.66 and 96.55±0.45, 85.71±0.79, 82.14±0.76 at 50, 25 and 12.5 mg/ml respectively was achieved with no significant difference ($P<0.05$) in egg hatchability with the positive control. The highest larvicidal mean percentage of 100.00±0.00 was achieved by *R. apetalus* and *H. abyssinica* across the three dosages with no significant difference between them and albendazole. GC-MS analysis revealed compounds such as terpenoids were conspicuously present which accounted for some of the activity. This observation lenders support to the traditional use of the plant extracts for the treatment of suspected helminthic infections.

Key words: *Haemonchus contortus,* anthelmintic, essential oil, monoterpenoids, terpenes.

INTRODUCTION

Agriculture in Kenya remains the backbone of the economy playing crucial roles, especially in the rural economy. It plays a very critical role in the provision of employment opportunities to citizens employing more

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than 40% of the country's population with the bulk being Kenyans living in the rural areas where more than 70% of them rely on agriculture as a source of income. The agricultural sector contributes to 65% of the total exports earnings made by the country (Kenya at a Glance | FAO in Kenya | Food and Agriculture Organization of the United Nations, 2017). The livestock industry accounts for 45% in the agricultural industry, and comprises of cattle, goats, sheep, and camels among others.

Helminthiasis which is as a result of infestation by helminths continues to be a major challenge in the productivity of farm animals all around the planet. Infestation by gastrointestinal parasites has become a major factor limiting productivity in livestock in Kenya. This is further compounded by resistance to currently used anthelmintic drugs (Shalaby, 2013).

Chemotherapeutic agents remain to be mainstay in the control of helminthiasis. These agents also have challenges associated with their use such as toxicity, increased cost of production, existence of drug residues in the animal products and the environment, cases of non-adaptability of the drugs and non-availability of the drugs in the remote parts of the country. This calls for the search of alternative control measures to deal with challenges of helminth infestation in livestock (Rajeswari, 2014). Plants have been used for the prevention and treatment of various diseases in humans and animals from time immemorial with more that 80% of drugs that are currently being used derived from plants or inspired by a natural compound (Selamoglu, 2018).

Haemonchus contortus also known as the Barber's Pole Worm, is one of the most prevalent and pathogenic nematodes affecting small ruminants, such as sheep and goats. These parasites adversely affect production due to high rates of mortality in the infected animals especially during the rainy seasons (Rajeswari, 2014). Parasitism by the gastrointestinal nematodes in ruminants and the emergence of resistance among these parasites has been documented across the globe (Kamaraj et al., 2011).

In Kenya multiple resistance to albendazole, levamisole, thiophanate and ivermectin that is orally administered has been documented in *H. contortus* isolated from a farm where resistance to benzimidazole had earlier been identified (Waruiru et al., 1997). Gastrointestinal nematodes affecting both goats and sheep that are resistant to ivermectin and febendazole have been identified in the coastal region of Kenya (Mwamachi et al., 1995).

Traditionally among the Meru community plants have been used in the treatment of various ailments afflicting the human population and the livestock as well. This knowledge has been passed on from one generation to another.

However, only a handful of researchers have since documented this knowledge with no single study documenting the effectiveness of these plants against helminths. Since there is no scientific information to validate this assertion, this study documents any effectiveness of the study plants against *H. contortus* and provide insights that are valuable in the advancement of modern medicine.

This study aimed to evaluate the *in vitro* anthelmintic properties of selected plants that have been commonly used in the treatment of suspected helminthic infestations among the Meru community.

MATERIALS AND METHODS

Collection and identification of the plant specimen

Bridelia micrantha, Aframomum zambesiacum, Hagenia abyssinica, Rubus apetalus, Thespecia garckeana, Physalis peruviana and Caesalpina volkensii used in this experiment were collected from Tharaka Nithi County with assistance from the local traditional medicine people who have used these plants traditionally to treat suspected helminthic infections. They were collected in the month of March where the temperatures range were 26 to 27ºC and an average annual precipitation of 147.98 mm. The plants were identified and authenticated with the help of botanists at the University of Nairobi, Botany Department and voucher specimens were deposited at the University of Nairobi herbarium.

Preparation of plant extracts

The plant materials that included the leaves of *B. micrantha*, *R. apetalus*, *P. peruviana* and *C. volkensii,* bark of *T. garckeana*, flowers of *H. abyssinica* and seeds of *A. zambesiacum* were collected and dried at room temperature. They were then ground into a fine powder using an electric grinder. 100 grams of the powdered material was soaked in 500 ml absolute methanol at 25°C for three days. The mixture was then shaken for 2 h at 200 rpm followed by centrifugation for 20 min at 4000 rpm at 4°C. The resultant supernatant was filtered through the Whitman filter paper 25 mm and then the methanol evaporated using the rotary evaporator. Finally, the accrued extracts were re-suspended in dimethyl sulfoxide before use.

H. contortus eggs and larvae

H. contortus eggs were obtained from faecal samples collected from farmers' sheep in Muguga Sub-County in Kiambu County. After collection the faecal samples were stored in polythene bags and transported to the Kenya agricultural and livestock research organization (KALRO) helminthology lab for extraction. The extracted eggs were used in the egg hatch assay while the larvae obtained after incubation of the eggs were used in the larvae development assay.

Egg hatch assay test

The procedure for the test adhered to recommendations by World Association for the Advancement of Veterinary Parasitology (WAAVP, 1992). Samples that were fresh and which contained at least one hundred eggs per gram from sheep were subjected to homogenization in tap water. They were then used to utterly fill up 100 ml bottles, hence rendering them anaerobic (von Samson-Himmelstjerna et al., 2009).

Eggs were then extracted through sieving, centrifugation, and flotation in 4 molar sodium chloride according to protocol by Števo and Cagan (2012). The eggs were then washed and suspended in tap water at a concentration of 100 eggs/ml. The suspension containing the eggs was subsequently inspected microscopically to ascertain commencement of embryonation. Each sample was tested at least in triplicates and negative control samples composed of the eggs and water and albendazole (Albafas, Norbrook, Kenya) for positive control were used.

The egg suspension was then placed in wells in a 24 well culture plate and then 10 mg of the extract from the different plants added. The plates were sealed to inhibit their drying out then incubated for 48 h at 25°C. This was followed by the addition of a drop of Gram's iodine to stop the assay (von Samson-Himmelstjerna et al., 2009). At least one hundred eggs and larvae were counted from each well and to fit the achieved dose-response data by a non-linear regression, and one way ANOVA followed by Tukey's test pairwise comparison to analyse the data on the percentage inhibition in hatching that occurred.

Larval development assay

The eggs were extracted and incubated in a 24 well micro-titre plate for 7 days at 25°C. A wet sponge was placed under the plate and the system covered with a pouch in order to prevent dehydration. After hatching, the wells were supplemented with growth nutritive medium containing 20 µl yeast extract and normal saline was added to the negative control well (Heim et al., 2015). The plant extracts were introduced into the wells of a micro titre plate after which at least one hundred living *H. contortus* larvae were introduced to each well and the plates were then returned to the incubator for six days. On the seventh day, all the larvae were counted under a microscope using an eel chamber as either living third stage larvae (L3) or dead larvae. This was conducted at least in triplicates for each plant extract. The negative control samples composed of the larvae and water and albendazole (Albafas, Norbrook, Kenya) for positive control were used.

Statistical data analysis

The results were reported as mean ± SEM (standard error of mean) and analysed with one-way analysis of variances (ANOVA) and Tukey's as the post hoc test was used to analyse the data acquired from the anthelminthic studies. Minitab version 17.0 was used in the analysis. The analysis was conducted at a significance level of $P < 0.05$.

RESULTS

Effects of the plant extract on *H. contortus* eggs hatching

Methanol extracts of *P. peruviana* and *R. apetalus* achieved the highest mean hatch inhibition of *H. contortus* eggs of 95.24±0.54, 90.00±1.00, 88.24±0.66 and 96.55±0.45, 85.71±0.79, 82.14±0.76 at 50, 25, and 12.5 mg/ml (Figure 1), respectively. Similarly, *H. abyssinica* had high mean inhibition percentages of 94.12±0.52 and 82.14±0.76 at 50 and 25 mg/ml (Figure 1), respectively. *C. volkensii* also achieved a mean egg hatch inhibition of 95.00±1.53, but only at the highest concentration of 50 mg/ml (Figure 1). There was no significant difference ($P<0.05$) in the mean percentage inhibition between the positive control (albendazole) and *P. peruviana, R. apetalus, C. volkensii*, and *H. abyssinica* at 50 mg/ml (Figure 1).

Effects of the plant extracts on *H. contortus* larval development

Methanolic extracts of *R. apetalus* and *H. abyssinica* had highest mean percentage larvicidal activity of 100.00±0.00 across the three dosages for the *H. contortus* larvae (Figure 2). There was no statistically significant difference between the two extracts and the positive control and between the plant extracts at this activity as well as the other plant extracts as shown in Figure 2. It was also recorded that at 50 mg/ml, *P. peruviana* and *C. volkensii* both had a mean percentage larvicidal activity of 100.00±0.00; hence, there was also no significant difference between the two and the positive control (Figure 2). At higher dilutions, the aforementioned plant extracts exhibited lower larvicidal (Figure 2).

Phytochemical screening for the plant extracts by Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS analysis on the methanolic extracts of the seven plants revealed the presence of monoterpenoid phenols such as 5-methyl-2-propan-2-ylphenol (thymol), 5-methyl-2-propan-2-ylcyclohexan-1-ol (menthol), 2-methyl-5-propan-2-ylphenol (carvacrol), 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (Borneol), 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (α pinene), 1,7,7-trimethylbicyclo[2.2.1]heptan-2-one (camphor), 3,7,7-trimethylbicyclo[4.1.0]hept-3-ene (3-carene), 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane (eucalyptol), 6,6-dimethyl-2-methylidenebicyclo[3.1.1]heptane (β pinene) and 4-methyl-1-propan-2-ylcyclohex-3-en-1-ol (terpinen-4-ol) which could be contributing to the anthelminthic activity observed among the plants in this study (Table 1). Also contained in the extracts are other classes of secondary metabolites which include sesquiterpenes, triterpenes, fatty acids, sterols, straight chain alkanes, norterpenes, 1-benzofurans, phenols, aromatic ketones, aromatic alcohols, aromatic hydrocarbons, monoterpenes, monohydroxyacetophenone, and vitamin E (Table 1).

DISCUSSSION

In particular, the methanolic extracts of *P. peruviana, R. apetalus, B. micrantha, C. volkensii* and *H. abyssinica* exhibited high activity while *T. garckeana* and *A. zambesiacum* exhibited moderate activity on egg hatch assay as well as larval development. There was no

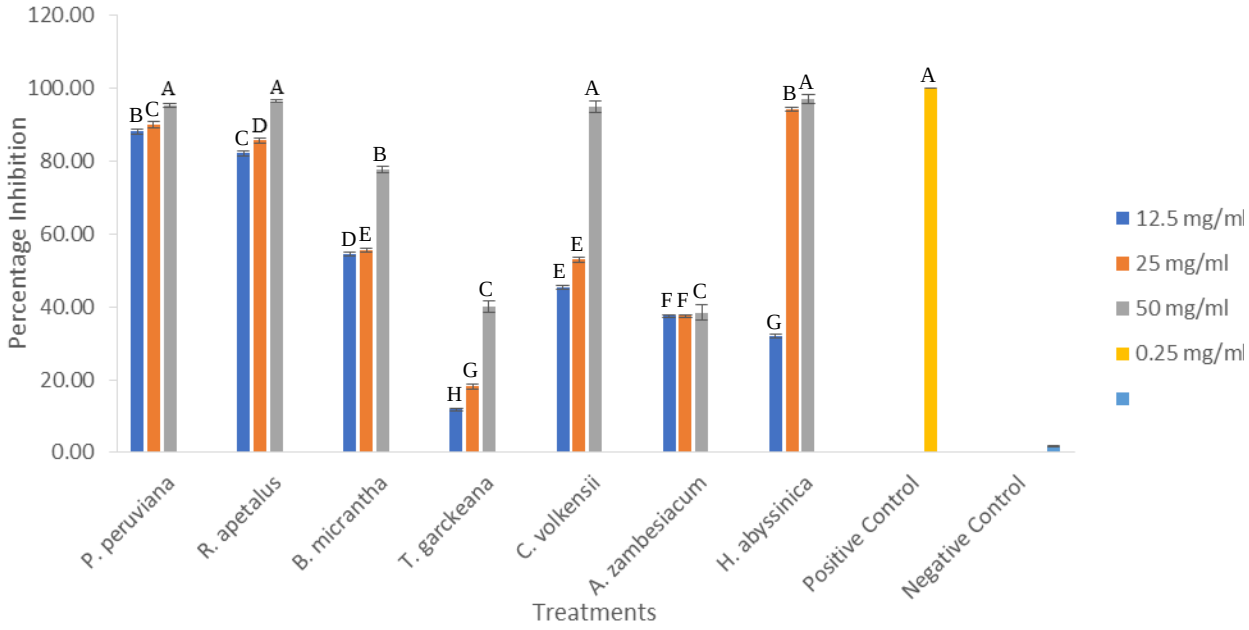

Figure 1. Mean percentage hatch inhibition of *H. contortus* eggs at varying methanolic plant extract concentrations. Values are mean ± SEM. The means that do not share letters are significantly different, Tukey's (P<0.05).

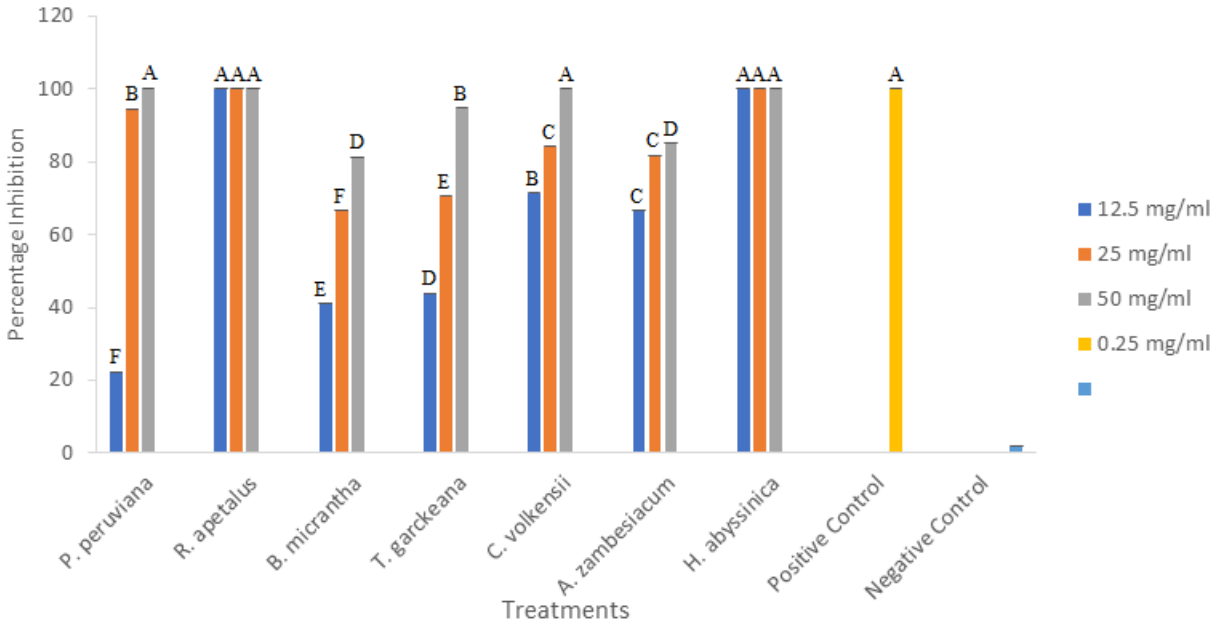

Figure 2. Mean percentage larvicidal activity against *H. contortus* larvae at varying methanolic plant extract concentrations. Values are mean ± SEM. The means that do not share letters are significantly different, Tukey's (P<0.05).

statistically significant difference (P>0.05) with the conventional drugs. The activity observed against *H. contortus* eggs and also larvae was dose related.

All the aforementioned plants were determined to contain saponins, alkaloids, tannins, terpenes and phenols. These secondary metabolites could account in part for the anthelminthic activity observed among the plants used in this study.

In particular, 3-Cyclohexene-1-methanol, alpha..Alpha.4-trimethyl- and 3-Cyclohexen-1-ol, 4-methyl-1-1-methylethyl)- are terpineols that were identified in the extract of *A. zambesiacum.*

Table 1. Phytochemicals found in the plant extracts after screening by gas chromatography - mass spectrometry (GC-MS).

No.	Phytochemical	Plant extracts						
		P. peruviana	***R. apetalus***	***B. micrantha***	***T. garckeana***	***C. volkensii***	***A. zambesiacum***	***H. abyssinica***
1	Monoterpenes	+	+	+	+	+	+	+
2	Sesquiterpenes	-	-	-	-	-	+	+
3	Triterpenes	+	-	-	-	+	+	-
4	Fatty Acids	+	+	+	+	+	+	+
5	Phenols	+	-	+	+	+	+	+
6	Sterols	+	-	-	-	-	-	-
7	Straight Chain Alkanes	+	-	+	+	+	+	+
8	Norterpenes	-	+	-	-	-	-	-
9	1-Benzofurans	-	-	-	-	+	+	-
10	Aromatic Ketones	+	-	-	-	+	-	-
11	Aromatic Alcohols	-	-	-	-	+	+	-
12	Aromatic Hydrocarbons	+	-	-	-	+	+	+

+ = Presence; − = Absent.

Terpineols have been shown to have anthelminthic properties and are known to inhibit hatching of helminth eggs (Grando et al., 2016; Mirza et al., 2020).

Terpineols have also been documented to inhibit hatching and migration of *H. contortus* larvae (Grando et al., 2016). However, the mode of action by which these secondary metabolites act is yet to be elucidated.

Terpenoids have been reported to harbour insecticidal activity where they have been associated with the inhibition of growth, maturation damage and decreased reproductive ability (Carvalho et al., 2012), which result in mortality in the insects.

B. micrantha, P. peruviana, A. zambesiacum and *T. garckeana* were all determined to contain Phenol, 2-methyl-5-(1-methylethyl)- which has been documented to be 100% effective in the egg hatch assay involving *H. contortus* at a dose of 2 mg/ml (Andre et al., 2016a).

Thymol a monoterpenoid phenol and a derivative of cymene which was present in *P. peruviana, A. zambesiacum, T. garckeana* and *C. volkensii* is known to possess various properties which include being an antioxidant, scavenging for free radicals, anti-inflammatory, antispasmodic, antibacterial, antifungal, antiseptic and analgesic (Nagoor Meeran et al., 2017). Thymol at a dose of 4 mg/ml was observed by Andre et al. (2016b) to inhibit hatching of *H. contortus* eggs by 98%. The ovicidal activity of thymol is attributed to the presence in its structure of the hydroxyl radical. It has been hypothesized that its mechanism of action against hatching entails either preventing changes in the permeability of the egg shell by binding to the lipoproteins of the membranes of the egg (Perry, 2002) or through binding competitively to hatching factors in the egg shell resulting in the altering of the process of hatching (André et al., 2017).

Carvacrol contained in *P. peruviana* is a monoterpenoid that has been found to harbour several pharmacological actions among them anthelmintic (Zhu et al., 2013), acaricidal (Costa-Júnior et al., 2016), harbour activity against *Leishmania infantum* and also *Trypanosoma cruzi* (Escobar et al., 2010). It has been established that at a concentration of 2mg/ml carvacrol was 100% effective against hatching of *H. contortus* eggs (Andre et al., 2016a). The ovicidal activity of carvacrol is attributed to the presence of the phenolic group in its structure. This radical has been postulated to inhibit enzymes such as the proteases, lipases, chitinases, beta glucosidase and leucine aminopeptidase which are responsible for hatching of the eggs (Andre et al., 2016a). This mode of action is similar to that of polyphenols and tannins that contain in their

structure the hydroxyl radical (Vargas-Magaña et al., 2014).

H. abyssinica and *R. apetalus* yielded statistically significant ($P<0.05$) inhibition of *H. contortus* larval development across the three concentrations tested. The most effective plant extracts were consistently found to contain different essential monoterpenoid oils which could perhaps explain the activity as these secondary metabolites have been shown to have different and unique targets and mode of action which include acting on proteins responsible for various functions in the parasite such as receptors.

Thymol has also been documented to affect the organization and the electrostatic properties of the membrane surface and this in turn changes the permeability and inhibits the activity of the membrane proteins such as the ATPases (Nagoor Meeran et al., 2017). It has also been established to penetrate the cuticle of the helminths and cause internal ultrastructural lesions (André et al., 2017).

P. peruviana was determined to contain monoterpenoid essential oil carvacrol which just like thymol is effective against nematodes and it has been documented by Kong et al. (2007) to have very strong nematocidal activity. It was found to be very effective against sheep gastrointestinal nematodes, *H. contortus* and *Bursaphelenchus xylophilus* nematode found in pine wilt (Kong et al*.,* 2007). The activity of thymol and carvacol is attributed to their ability to trigger a signalling cascade that leads to eventual demise of the nematodes through interaction with a TyrR like SER-2 receptors (Lei et al., 2009). The interaction of thymol and carvacrol with the SER-2 results in the altering of the functions of the receptor.

Carvacrol results in changes in the cuticle and the intestine of the L3 larvae form of *Anisakis simplex* and its efficacy against helminths is credited to the presence in its structure of the phenolic group which causes helminth excretory cell lysis and alterations to the cuticle (Hierro et al., 2004). This monoterpenoid has effects on the membrane of the bacteria, consisting of the phenol group connection with the amine and hydroxyl groups of the membrane proteins in bacteria. The interaction between the hydroxyl radical and the membrane proteins affect the lipid layer stability and this increases the passive flow of the protons across the membrane resulting in the altering of the permeability and eventual cell death (Andre et al., 2016a).

Thymol and carvacrol have been established to result in the damage of the cuticle and the digestive apparatus in the larvae of *Anisakis* (Giarratana et al., 2014). Besides the changes to the cuticle, carvacrol is known to be neurotoxic to the nematode *Caenorhabditis elegans* where it interacts with the SER-2 tyramine (Lei et al., 2010). The changes in the cuticle and neurotoxicity caused by carvacrol have the possibility of interfering with the permeability of the cuticle and motility hence impeding maintaining of homeostasis within the parasite (Andre et al., 2016a). The cuticle forms a barrier that protects the parasite and is also involved in the metabolic exchanges.

In addition to cuticular changes carvacrol also results in changes in the reproductive tract of parasites. The structural alterations in the external reproductive organs of the female parasite also affects reproduction in the parasite and reduces the production of eggs by the parasite (Andre et al., 2016a). Carvacrol in acidic pH values has been established to have antifungal activity (Chavan and Tupe, 2014) and has also been documented to be effective against different stages of the *H. contortus* (Andre et al., 2016a).

Most of the studied plants were shown to have antihelmintic effects; in particular, methanolic extracts of *P. peruviana, R. apetalus, C. volkensii*, and *H. abyssinica* produced effects comparable to the conventional drug. The plant extracts that exhibited significant antihelmintic activity contained 3-Cyclohexene-1-methanol, alpha.alpha.4-trimethyl-, 3-Cyclohexen-1-ol, 4-methyl-1-1-methylethyl)-, phenol, 2-methyl-5-(1-methylethyl)-, thymol, and carvacrol which most likely were responsible for the activity.

The plants that were studied should continue to be used for control of helminth parasites in Meru and their use promoted as an adjunct to conventional anthelmintic control. Further studies should be carried out to elucidate the individual anthelmintic activity of the various secondary metabolites contained in the plants used in this study. The modes of action by these metabolites should also be investigated for use as potential novel anthelminthic drugs and similar plants as was used in this study but from a different geographical location should be studied to determine any differential effects. Toxicity studies on the most effective plants of this study ought to be conducted so as to develop confidence in the use of the herbal remedies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the whole staff at the Department of Helminthology at Kenya Agricultural and Livestock Research Organization (KALRO) for their exceptional guidance and technical support. They also thank botanists at the University of Nairobi, Botany Department for participating in the identification of the plant specimens.

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