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Anthelmintic and Antibacterial Activity of *Hagenia abyssinica* (Bruce) J.F. Gmel (Rosaceae)

E.W. KARUMI[1]*, C.K. MAITAI[1], F.A. OKALEBO[1], N.N. MUNGAI[1], S.N. NDWIGAH[2], P.C. MUTAI[1] AND N.A. MUKUNGU[1]

[1]Department of Pharmacology and Pharmacognosy, and [2]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Nairobi, P.O. Box 19676-00202, Nairobi, Kenya.

Organic solvent extracts of the stem bark of *Hagenia abyssinica* Bruce J.F. Gmel (Rosaceae) were screened for anthelmintic and antibacterial activity. The methanol and dichloromethane/methanol extracts exhibited anthelmintic activity when tested using *Panagrellus redivivus* model. Further, the stem bark methanol extract showed significant but weaker activity than levamisole against *Caenorhabditis elegans* species. The petroleum ether and the dichloromethane/methanol extracts exhibited significant antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* at a concentration of 50 mg/ml, but no antifungal activity.

Key words: *Hagenia abyssinica*, anthelmintic, antibacterial

INTRODUCTION

Hagenia abyssinica (Bruce) J.F. Gmel (Rosaceae), known commonly as African redwood, is a monotypic genus in the Rosaceae family. It is a flowering plant which is native to the Afromontane regions of Central and Eastern Africa. In Kenya, it is mainly dominant in the woodland zone just above the bamboo and also in moist forest below bamboo [1, 2]. It is a slender tree, 5 to 25 m tall, with a short trunk and thick branches; with branchlets covered in silky brown hairs and ringed with leaf scars. Its bark is thick, brown or reddish-brown and readily peels. It has compound leaves with 3-6 pairs of leaflets plus a terminal leaflet, each about 10 cm long.

The leaf margins of *Hagenia abyssinica* are serrated and fringed with long hairs and the leaf stalks are 12 cm long, with expanded wings formed from the stipules, and are densely hairy on the underside. The flowers are greenish, or white, turning reddish with maturity and they form handsome multibranched, terminal, drooping panicles up to 60 cm long and 30 cm wide. They are polygamo-dioecious with pinkish-red female heads while the male heads are feathery orange-buff to white. The fruits are usually small, dry, winged, asymmetric, single seeded, brown syncarp with a single ovoid carpel and fragile pericarp [1, 2].

The dried and pounded female inflorescence of *Hagenia abyssinica* is used as an anthelmintic in ruminants and in humans. The roots of the plant are cooked with meat and the soup drunk for malaria; whereas the pounded bark is drunk in cold water as a remedy for diarrhea and stomach ache [3]. The powdered seeds are applied on wounds and cuts of both humans and livestock [4]. The plant flowers are also reported to cause abortions [5].

MATERIALS AND METHODS

Plant collection and identification

The stem barks of *Hagenia abyssinica* were collected from the Aberdare Ranges, about 100 km from Nairobi, in Kiburu Forest Station and surrounding areas in September 2009. The plant was identified on site and the identity of the plant confirmed by Mr. Mathenge at the National Museums of Kenya Herbarium. A voucher specimen designated HAP0036 was deposited at the School of Pharmacy, University of Nairobi. The stem barks were dried at room temperature and ground into coarse powder.

Preparation of extracts

About 700 g of stem bark powder was Soxhlet extracted sequentially with petroleum ether, chloroform and methanol. Another 1000 g stem

*Author to whom correspondence may be addressed.

bark powder was extracted with a 50:50 methanol/dichloromethane mixture. The extracts were reduced to dryness *in vacuo*.

Isolation of compounds

The petroleum ether extract was fractionated on silica gel by gradient elution with n-hexane, dichloromethane, ethyl acetate and methanol. Fraction HA4 formed a crystalline deposit after four days. The deposit was washed with methanol and dissolved in chloroform and left overnight and recrystallised in acetone yielding 23 mg. The compound obtained was designated **KWE01**. Thin layer chromatography of the crystals was carried out and then further recrystallization carried out by dissolving in 10% v/v methanol in chloroform and slowly evaporating the solution.

Screening for anthelmintic activity

A qualitative assay to screen the extracts for activity against roundworms was carried out. *Panagrellus redivivus* served as the model organism [6]; 60% ethanol served as the negative control; while 160 mg/ml $CuSO_4$ served as the positive control. One hundred microlitres of worm suspension were placed in 96-well plate and five microlitres of each of the plant extracts were added to the wells other than the controls, and plate incubated for 4 h. The plate was observed from beneath by holding a light to it. If worms were killed by the plant extract, they stopped wriggling and collected at the bottom of the u-shaped well. Active extracts were subjected to further assays using *Caenorhabditis elegans* [7].

Anthelmintic assays using *Caenorhabditis elegans*

Caenorhabditis elegans worm culture was synchronized by centrifuging at 1000 rpm at room temperature for 30 s, discarding the supernatant and adding alkaline bleach. The worms were then washed in M9 buffer to remove the alkaline bleach and resuspended in M9 buffer for 2 days. The media was prepared by addition of 7 ml OP50 to 62.3 ml of sterile M9 buffer. To the mixture, 0.7 ml ampicillin was added and 2 ml of the media were pipetted into each well of a 48-well plate.

In the whole worm assay [7], the worm suspension was prepared by introducing the synchronized cultures into nematode growth media plates with OP50 and incubated at room temperature for 44 h. Fifty microlitres of the worm suspension were pipetted into each well and the worms in each well counted. Five microlitres of the test compound (10 mM for pure compounds and 20 mg/ml for extracts) were introduced into the wells and the plates incubated for 24 h at 22°C. The anthelmintic activity of test extracts against the model organism was determined by enumeration of living and dead nematodes under a microscope and the percentage of worms surviving after 24 h incubation period recorded.

In the cut worm assay [8], adult worms were picked and transferred onto a slide containing artificial perienteric fluid (APF). Extracts were diluted to a final concentration of 1 mg/ml in dimethylsulfoxide (DMSO). The positive control (levamisole) was dissolved in APF to a final concentration of 100 µM. The negative control was 5% DMSO in APF. The worms were cut transversely between the anterior and middle segments, making sure that the cut was clean, and then transferred into 100 µL of the test solution in APF. The worms were observed under the microscope and the time taken to cause paralysis recorded.

The time to paralysis was analyzed using Survival Analysis Model. The software used to achieve this was STATA® Version 12 (Corp, Texas, USA). The hazard ratio (HR) and their 95% confidence intervals (C.I.) were displayed. P values < 0.05 were regarded as statistically significant.

***In vitro* testing for antimicrobial activity**

Bacterial test microorganisms namely *Staphylococcus aureus* (NC 07447), *Escherichia coli* (ATCC 25922) and *Bacillus subtilis* (NC 08241) were subcultured onto slants of Tryptone Soy Agar and incubated at 37ºC for 24 h while the fungal test microorganisms, *Candida*

albicans (NCPF 3179) and *Saccharomyces cerevisiae* (ATCC 9763) were subcultured on Sabourauds Dextrose Agar at 25ºC for 48 h. All the plant extracts were weighed and dissolved in DMSO to a final concentration of about 50 mg/ml solutions. Erythromycin and nystatin solutions were used as standards at concentrations of 10 µg/ml and 100 µg/ml, respectively.

The plate agar diffusion method according to Kavanagh was adopted to assess the antibacterial activity of the prepared extracts [9]. Nutrient agar was prepared according to manufacturer's instructions. It was sterilized in a steam autoclave at 121°C for 20 min and left to cool to about 50°C on the bench. A loopful of the standardized microorganism stock suspensions was thoroughly mixed with 100 ml of the sterile nutrient agar to produce an inoculum of approximately 1×10^6 colony forming units per ml. Twenty millilitres of the inoculated nutrient agar were distributed into sterile petridishes to form uniform thickness of about 3 mm. The agar was left to set. In each of these plates, 8 wells of 8 mm in diameter were cut using a sterile cork borer and the agar discs removed. Alternate wells were filled with 50 µl of each extracts, DMSO and standards using microtiter pipettes and allowed to diffuse at room temperature for 0.5 h in a laminar flow cabinet. The plates were then incubated in the upright position at 37ºC for 18 h. Two replicates were carried out for each extract against each of the test organism. After incubation the diameters of the zones of inhibition were measured, averaged and the mean values tabulated.

RESULTS AND DISCUSSION

Anthelmintic activity

The methanol and the dichloromethane/ methanol crude extracts exhibited anthelmintic activity while the petroleum ether and chloroform extracts showed no activity. The isolated compound identified as β-sitosterol exhibited no activity. This suggests that anthelmintic activity resides in the polar components since non-polar extracts showed no activity.

Whole worm assay

At the beginning of the assay, there were 13 worms in each well. The anthelmintic activity of *H. abyssinica* methanol extract was determined by enumeration of living and dead nematodes using a microscope. The light stimulation during the microscopic investigation induced movement of living nematodes where agile nematodes were considered alive and immotile ones considered dead. 5% DMSO was the negative control and was not expected to affect the viability of worms. The buffer in which the worms were maintained was also not expected to affect their viability. However, 12% of the test worms died in the buffer and 23% died in the negative control. This may have resulted from the drying effects caused by light during microscopic investigation.

Table 1 shows the percentage of live worms after a 24 h incubation period post-treatment. Incubation with levamisole resulted in 85% death of all the worms while incubation with the test drug resulted in 67% death. This shows that *H. abyssinica* stem bark methanol extract has anthelmintic activity but it is less active, at 20 mg/ml concentration, than levamisole.

Table 1: Percentage of worms alive after incubation at room temperature for 24 h

Test extract/Compound	Adult worms alive after 24 h (%)
M9 Buffer	88
5% DMSO	77
Levamisole (10 mM)	15
*H. abyssinica**	33

*20 mg/ml methanol extract of *H. abyssinica*.

Cut worm assay

The results obtained for cut worm assay are as presented in Table 2. The time taken for the worms to be completely paralyzed was shortest for the positive control, levamisole, and longest for the negative control. *Hagenia abyssinica*

methanol extract showed moderate activity with the time to paralysis being shorter than that of the negative control except for worm number five which can be considered as an outlier. The mean time taken to paralyze the worms by *H. abyssinica* methanol extract was 1.7 times less than that taken by the negative control, DMSO. However, the evidence to support that was weak (HR = 1.73, 95% C.I. = 0.51-5.84, p = 0.38).

Table 2: Time taken for complete paralysis of *Caenorhabditis elegans* worms after administration of drug compounds/extracts

	Time (min)						
Worms	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**
Hagenia abyssinica methanol extract (1 mg/ml)	5.52	2.10	2.52	5.38	8.07	7.50	5.18
Levamisole 100 µM (Positive control)	1.50	1.55	1.57	1.37	3.00	3.20	2.03
5% DMSO in APF (Negative control)	6.40	6.35	6.41	7.02	6.38	12.5	7.51

Antimicrobial activity

Table 3 presents the mean diameters of the zones of inhibition produced by the stem bark extracts of *H. abyssinica* in comparison with standard antimicrobials. The extracts were used at a concentration of 50 mg/ml. There was a significant difference in antibacterial activity for extracts obtained using different solvents. The petroleum ether and dichloromethane/methanol extracts exhibited a high degree of antibacterial activity against all the tested bacteria. The dichloromethane/methanol extract showed activity against *S. aureus* (with a zone of inhibition of 19 mm), *E. coli* (20 mm) and *B. subtilis* (18 mm) whereas the petroleum ether extract showed activity against *S. aureus* (with a zone of inhibition of 17 mm), *E. coli* (16 mm) and *B. subtilis* (16 mm) while that of erythromycin against *S. aureus* at a concentration of 0.01 mg/ml was 22 mm. The methanol extract exhibited no antibacterial activity.

The antibacterial activity against both the Gram-positive and Gram-negative bacteria exhibited by the dichloromethane/methanol and the petroleum ether extracts could be due to the phenolics, alkaloids or the phytosterols present in the plant. All four extracts did not exhibit any antifungal activity against the test microorganisms. The results for antifungal activity are unlike what was obtained in a previous study by Abera *et al.* in which ethanolic and aqueous extracts of *H. abyssinica* showed mild activity against *Colletotrichum kahawae*, a fungus which causes coffee berry disease [10].

Table 3: Diameters of the zones of inhibition produced by solvent extracts of *Hagenia abyssinica* against test microbes

Test solution	Diameters of zones of inhibition (mm)				
	Staphylococcus aureus	***Escherichia coli***	***Bacillus subtilis***	***Saccharomyces cerevisiae***	***Candida albicans***
Dichloromethane/ methanol extract	19.0	20.0	18.0	8.0	8.0
Chloroform extract	8.0	8.0	9.0	8.0	8.0
Methanol extract	8.0	8.0	8.0	8.0	8.0
Petroleum ether extract	17.0	16.0	16.0	8.0	8.0
Erythromycin 0.01 mg/ml	22.0	20.0	20.0	-	-
Nystatin 100 mg/ml	-	-	-	10.0	12.0

Note: Solvent extracts = 50 mg/ml; Zones of inhibition include 8.0 mm well diameter.

CONCLUSION

The methanol and dichloromethane/methanol stem bark and flower extracts exhibited anthelmintic activity while the petroleum ether and chloroform extracts showed no activity. This shows that anthelmintic activity resides in the polar components. β-sitosterol was isolated, for the first time, from stem bark of *H. abyssinica* but was found to have no activity against the bacteria and fungi species used in the experiment. Petroleum ether extract and dichloromethane/methanol extracts showed significant activity against all the bacterial microorganisms tested namely *Staphylococcus aureus, Esherichia coli* and *Bacillus subtillis.* The results support to a certain degree the traditional medicinal uses of the plant.

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