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Anthelmintic Screening of Phytomedicines using *Haemonchus placei* Adult Motility Assay

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: The leaf extract of *S. mombin* was previously shown to be active against gastrointestinal nematodes (egg and infective larvae) in sheep.
Objective: To examine *S. mombin* extracts against adult *H. placei*, a cattle parasite and optimise the adult worm motility assay.
Materials and Method: Levamisole hydrochloride was used to optimise incubation time in the assay protocol. Adult worms (ten) were incubated, in duplicate, with standard drug solutions in either phosphate-buffered saline, pH 7.4 (PBS-7.4) or normal saline (NS) for 3h. Worms were afterwards examined and classified as dead or paralysed. The activities of three *S. mombin* leaf extracts (AH-H01, AH-A02, AH-W03) were investigated and median lethal concentration (LC_{50}) was estimated by sigmoidal curve-fitting analysis.
Results: Three hours incubation time was found optimal. Significantly, NS was better than PBS-7.4 in solvating plant extracts, which gave LC_{50} values of (AH-H01; 104 mg/mL), (AH-A02; 30.5 mg/mL) and (AH-W03; 56.27 mg/mL) respectively.
Conclusion: *S. mombin* extracts are active against adult *H. placei* and the optimised assay enhanced sample throughput in anthelmintic phytomedicine screening.

Keywords: Screening assay, Heamonchus placei, Anthelminitics, Spondias mombin, Plant extracts

INTRODUCTION

The increasing incidence of drug resistance in gastrointestinal nematodes of livestock warrants the development of novel anthelmintics (James and Davey 2007 , Papadopoulos 2008, Zenebe et al., 2017). In recent times, plant-derived alternatives have become of serious interest because of concern over drug residues associated with prevalent use of synthetic anthelmintics. In addition, synergism between various secondary metabolites found in plant extracts is considered attractive to prevent ready development of resistance by helminths (Zhu et al., 2013, Novobilský et al., 2013).

Anthelmintic screening commonly uses egg hatch assay, infective larvae development assay, larval feeding inhibition assay or adult worm motility assay (Katiki et al., 2011, Ademola et al., 2009, Ademola et al., 2007, Ademola et al., 2004). Some attempts were made to develop high-throughput adaptable assays based on a real-time cell monitoring device (Smout et al. 2010) or real-time PCR technique (Subhadra et al., 2013). An *in- vitro* assay for screening anti-schistosomal drugs adopted fluorimetric analysis of resazurin as viability marker (Marxer, et al., 2012). The traditional methods have the advantages of low-cost and ease of implementation in resource-poor economies, where the need for alternative therapies to

limited and expensive synthetic drugs is more urgent and important. A particular *in vitro* anthelmintic screening assay (Sharma et al. 1971) has found application to the screening of medicinal plants for anthelmintic activity (Jabbar et al., 2007, Zenebe et al., 2017). The assay is based on adult worm motility of *Haemonchus contortus*.

A standard procedure for the assay typically uses 5 adult worms in triplicate in separate petri-dishes. Plant extracts are incubated with the worms, using phosphate buffered saline (PBS, pH 7.4) as incubation medium. The inhibition of motility and/or mortality of the worms were used as criteria for anthelmintic activity. The motility was recorded after 0,1,2,3 and 6h intervals. Finally, the treated worms were kept for 30 min in lukewarm fresh PBS, after each time interval, to observe possible revival of motility. Data analysis was done by probit transformation in order to transform a sigmoidal model to a linear relationship, and subsequent estimation of lethal concentration for 50% response (LC_{50}).

It was observed that the experimental procedure is rather time-consuming and thus with a low sample throughput. We therefore set out to optimize and validate the assay for anthelmintic screening. The streamlined method is expected to improve sample throughput by reducing cycle time. Secondly, the assay methodology is amenable to evaluation of anthelmintic small molecule drugs, phytochemicals, and phytomedicines that possess anthelmintic properties. In this paper, we report an initial adaptation of the method by Sharma et al., 1971, using levamisole as a model anthelmintic to optimize assay protocol against *Haemonchus placei* adult worm, which is a cattle parasite. Preliminary results guided optimization strategy and comparison of PBS and Normal saline (NS) as test medium.

The leaf extract of *Spondias mombin* was previously shown to be active against gastrointestinal nematode (egg and infective larvae) in sheep (Ademola et al., 2005). The optimised assay was used to investigate the activity of *S. mombin* extracts against adult *H. placei*.

METHODOLOGY

Plant material

The leaves of *Spondias mombin* (Anarcardiaceae) were collected from a tree growing around the Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria. Voucher specimen was deposited at the Forestry Research Institute of Nigeria, Ibadan, with a Voucher Specimen Number (FHI 108820).

Chemicals and Reagents

Sodium chloride (BDH, U.K), acetone, n-hexane (BDH, UK), potassium dihydrogen phosphate, dipotassium hydrogen phosphate,(Sigma, U.S.A), phosphate buffer saline (PBS,pH 7.4) was prepared as described in the United States Pharmacopoeia (Commission, 2000), Samtrex tablets (47.2 mg levamisole hydrochloride (equivalent of 40 mg levamisole base), Sam Pharmaceuticals Limited, Ilorin, Kwara State, Nigeria), levamisole hydrochloride (isolated in our laboratory).

Isolation of levamisole hydrochloride from tablets

The isolation procedure was developed by an adaptation of the monograph entry on identity test for levamisole hydrochloride in the International Pharmacopoeia, 4th edition (World Health Organization, Geneva, 2013).

Briefly, 20 tablets of levamisole hydrochloride were powdered in a mortar. A quantity of the powdered tablets containing the equivalent of 400 mg of levamisole was weighed and mixed with 30 mL of distilled water in a small beaker (50 mL). The mixture was vortexed and filtered. The residue was washed with 20 mL of water and the washings were added to the filtrate. To the combined filtrate, ammonia (6M) was added to make the solution alkaline (universal indicator, pH 8). It was then extracted with two quantities, each of 25 mL of dichloromethane. The dichloromethane extract was combined and evaporated to dryness. Hydrochloric acid (0.5 mL, 11.5 M) was added and heated on water bath to dryness. The residue was dissolved in ethanol, filtered and evaporated to dryness. The crude crystals were recrystallised from dichloromethane.

Collection of adult H. placei worms

Adult *H. placei* were obtained from the abomasums of infected cattle immediately after slaughter. The parasites were washed and kept in phosphate buffered saline (PBS, 7.4) for about 2 hours, when they were ready for use.

Optimization of screening assay

Anthelmintic profile of levamisole: adaptation of Sharma et al. 1971.

Five actively moving worms were placed in petri dishes with graded concentrations of levamisole hydrochloride (a model anthelmintic) ranging from 10.88 ng/mL to 651 ng/mL in phosphate buffer saline (PBS, pH 7.4). The test was set up in triplicates. The number of motile (alive) and immotile (dead) worms were counted after 1, 2, 3 and 6 hours of incubation in drug solution and 30 minutes of incubation in fresh PBS-7.4, after each time interval, to induce worm recovery.

Anthelmintic profile of levamisole: optimized assay protocol

Ten actively moving worms were placed in petri dishes, in duplicate, with graded concentrations of levamisole hydrochloride at 0, 1.25, 2.50, 5.0, 10.0, 20.0, 40.0 and 80.0 ng/mL in PBS-7.4. The worms were incubated at room temperature for 3 hours. At the end of the time interval, the worms were incubated for 30 minutes in fresh PBS 7.4 at 38 ºC and examined for death or paralysis by counting the number of motile (alive) and immotile (dead) worms. Worm motility was determined by sinusoidal motion of the worm in the incubation medium.

Anthelmintic profile of levamisole: normal saline as incubation medium

The assay was repeated as described under 2.5.2, but with the PBS-7.4 incubation medium replaced with normal saline.

Extraction

The air-dried plant was powdered with a laboratory blender and defatted by cold maceration in *n*-hexane for 24 hours. The hexane extract was concentrated in rotary evaporator under reduced pressure. Final drying was done in vacuum oven at 60ºC. The dry extract was labelled AH-H01. The defatted plant material was extracted by cold maceration with acetone for 24 hours. The acetone extract was concentrated in rotary evaporator under reduced pressure and finally dried in vacuum oven at 60ºC. The dry extract was labelled AH-A02. Another sample of defatted plant material was extracted with distilled water by cold maceration for 24 hours. The aqueous extract was concentrated first in rotary evaporator after addition of acetone to the aqueous extract. Final drying was done in vacuum oven at 60ºC (AH-W03). Extraction by solvents of varying polarity was meant to separately investigate plant constituents of varying structure and physicochemical properties, represented by the various extracts.

Effect of incubation medium on performance characteristics of screening assay

Standard solutions of extract AH-A02 were prepared by taking aliquot of stock solutions in 20% Tween 80, and using as diluent, normal saline and PBS-7.4. Ten actively moving worms were placed in petri dishes, in duplicate, with graded concentrations of AH-A02. The concentrations ranged from 0 -20 mg/mL in PBS-7.4, while the concentration ranged from 0 - 50 mg/mL in NS solution. (The difference in the concentration range was due to inherent difference in the solubilization capacity of the two diluents). The worms were incubated at room temperature for 3 hours. At the end of the time interval, the worms were incubated for 30 minutes in PBS 7.4 at 38 ºC and examined for death or paralysis by counting the number of motile (alive) and immotile (dead) worms.

Evaluation of assay in screening plant extracts containing structurally diverse molecules.

Stock solutions of the extracts were prepared as follows; 45 mg/mL in 30% tween 80 (AH-H01), 50 mg/mL in 20% tween 80 (AH-A02), 100 mg/mL in normal saline (AH-W03). Standard solutions of extract AH-H01, AH-A02 and AH-W03 were prepared in turn, by taking aliquots of stock solutions, and using normal saline as diluent. The concentrations ranged from 0 - 45 mg/mL, 0 - 50 mg/mL and 0 - 80 mg/mL for AH-H01, AH-A02 and AH-W03 respectively. The incubation of worms and data-acquisition was carried out as described under section 2.5.2 above.

Curve-fitting and statistical analysis

The concentration - response curve plotted mean number of dead worms against log concentration of the anthelmintic sample. Non-linear regression analysis fitted sigmoidal equation with a fixed slope of 1 (equation 1) for levamisole data. However, for the plant extracts which are expected to show variation in anthelmintic activity, a four logistic parameter sigmoidal equation (i.e. a variable slope sigmoidal equation, equation 2) was fitted to the data. The constraints set for the assay were; Bottom is constant = 0, Top is constant = 10. The best-fit LC_{50} was then calculated with the associated uncertainty expressed as 95% confidence interval, while the regression coefficient, R^2 measures the goodness-of-fit. All the analyses were performed by GraphPad Prism Version 4.03 (SanDiego, CA, USA).

2.10 Equations

i. Fixed slope equation

$$Y = Bottom + \frac{(Top - Bottom)}{(1+10^{((LogEC50 - X))})} \quad \ldots\ldots\ldots (1)$$

ii. Variable slope equation

$$Y = Bottom + \frac{(Top - Bottom)}{(1+10^{((LogEC50 - X)\times Hillslope))})} \quad \ldots\ldots (2)$$

(X = Log of concentration, Y = Biological response, Y starts at Bottom and goes to Top with a sigmoid shape, Top = the maximum response, Bottom = the baseline response, Hillslope = the slope, EC_{50} = drug concentration that provokes a response halfway between baseline and maximum response).

RESULTS

Anthelmintic profile of levamisole

The initial results of the assay procedure implemented by adapting the method of Sharma et al. 1971, is displayed in Table 1. It was observed that graded response to levamisole was only obtainable at concentrations between 0 - 80 ng/mL, beyond this range, all the worms were killed. It was also observed that 3 hours was a suitable incubation time because the response at 3 hours was comparable to the response at 6 hours for all practical purposes. The advantage of taking measurement at 3 hours is the significant savings in cycle time for every assay procedure. This feature is critical to improving the sample throughput of an assay intended for screening candidates in drug discovery programs. The optimized assay protocol adopted 10 worms per set up in duplicates with an incubation time of 3 hours. In addition, the worms were kept in fresh PBS -7.4 at 38ºC for 30 minutes to induce recovery of worms that were merely paralyzed rather than dead during incubation with the test drug. No specific temperature was specified in the earlier report that recommended use of lukewarm PBS solution to induce recovery of worms (Sharma et al. 1971).

The anthelmintic profile of levamisole is similar when either PBS-7.4 (Figure 1A) or Normal saline (NS) (Figure 1B) was employed as incubation medium. LC_{50} of 8.94 and 8.93 ng/mL were obtained in PBS-7.4 and NS respectively. The goodness-of-fit parameters represented by 95% confidence interval and regression coefficient R^2 were also found similar and reliable.

Table 1: Worm survival pattern for adult *H. placei* worms, after incubating the worms (5 per set up) in triplicate, in PBS-7.4 incubation medium, with graded concentration of levamisole.

Incubation time (h)	Mean number of dead worms (mean±SD) [Concentration, ng/mL]						
	651	326	163	81.5	40.8	20.4	10.88
0	0	0	0	0	0	0	0
1	5	5	5	4.33± 0.58	1.33± 1.53	0	0
2	5	5	5	5	4.66± 0.58	3.33 ±1.53	1.33± 0.58
3	5	5	5	5	5	3.67± 0.58	2.67± 1.15
6	5	5	5	5	5	5	3.33± 1.00

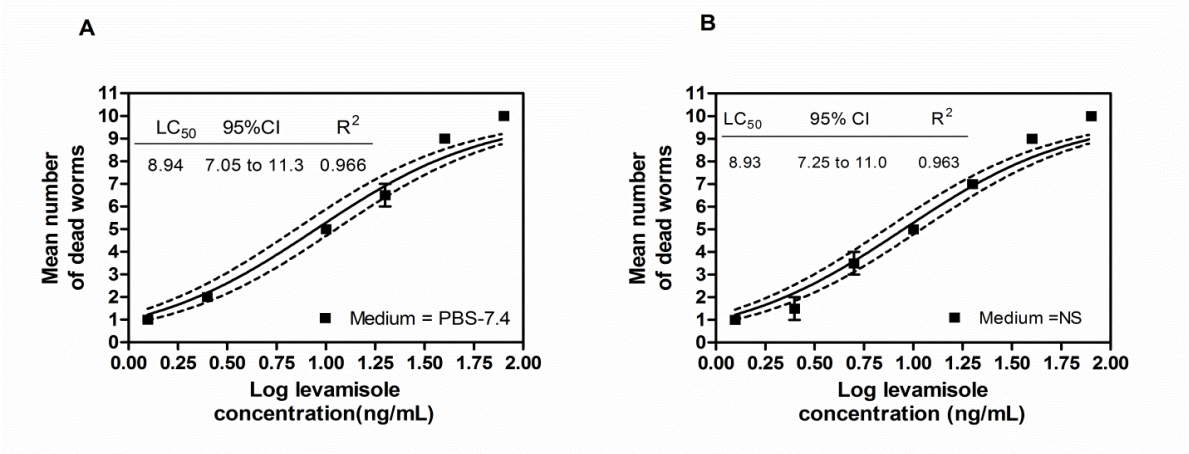

Figure 1: (A) Concentration-response curve of adult H. placei worm incubated with levamisole in PBS-7.4 incubation medium, using fixed slope sigmoidal model for curve fitting. (B) Concentration-response curve of adult H. placei worm incubated with levamisole in Normal Saline (NS) incubation medium, using fixed slope sigmoidal model for curve fitting. Constraints (Top is constant = 10, Bottom is constant = 0)

Effect of incubation medium on performance of assay

The relative usefulness of PBS-7.4 and NS as incubation medium for the anthelmintic assay was assessed by profiling the acetone extract (AH-A02) of *S. mombin* leaves, which was previously shown to exhibit anthelmintic activity (Ademola, et al. 2005). The dynamic range is wider in NS with an upper limit of 50 mg/mL, while PBS-7.4 has an upper limit of 20 mg/mL. Secondly, the 95% CI, a measure of the

reliability of best-fit assay parameters, obtained in NS is much narrower (28.8 to 32.2 mg/mL) than the value for PBS-7.4 (26.5 to 65.7 mg/mL). The best-fit LC_{50} value in NS is 30.5 mg/mL, as opposed to 41.7 mg/mL in PBS-7.4 (Figure 2). The result shows that using NS as incubation medium reported a lower LC_{50} value, which signifies greater potency for the extract, than what was reported with PBS-7.4 as incubation medium. The narrower 95% CI obtained in NS when compared with PBS also suggest greater reliability of measurement. The narrower dynamic range and fewer data points permissible in PBS-7.4 are responsible for the worse statistics associated with the assay results.

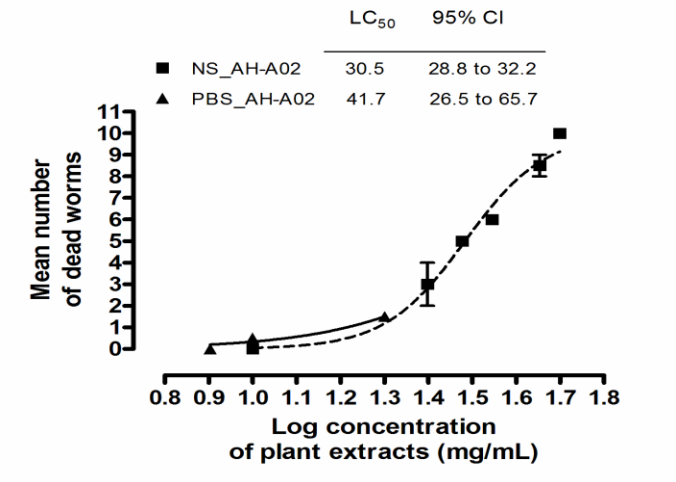

Figure 2: Comparison of concentration-response curves of adult H. placei worm incubated with acetone extract in normal saline (NS) versus PBS as incubation medium. The upper limit of the dynamic range for PBS is 20 mg/mL, which is much lower than the upper limit of 50 mg/mL obtained for NS incubation medium. The wider dynamic range and the narrower confidence interval obtained with NS as incubation medium underscores the greater solvating power of NS and the superiority of NS as incubation medium for the screening assay

Assay performance in screening structurally-diverse small molecules.

Typically, plant extracts are composed of structurally-diverse molecules ranging from highly lipophilic secondary metabolites that partition into *n*-hexane to very polar secondary metabolites that partition into water. Acetone of medium polarity will contain a more balanced mixture of polar and non-polar compounds. In order to assess the performance of the assay for screening plant extracts in early-stage drug discovery science, the anthelmintic profile of *n*-hexane (AH-H01), acetone (AH-A02) and aqueous (AH-W03) extracts were compared, using NS as incubation medium (Figure 3). The concentration-response curve of the *n*-hexane and aqueous extracts were both shifted to the right of the acetone curve, a demonstration of lower potency. Best-fit LC_{50} values rank the potency of the extracts as AH-A02 > AH-W03 > AH-H01 with 30.5 mg/mL, 56.2 mg/mL, and 104 mg/mL respectively. The predominantly lipophilic nature of molecules found in *n*-hexane extract is evident in the poor water solubility of the extract, and hence, low upper limit of the dynamic range (Figure 3). The best-fit, goodness-of-fit and other validation parameters for the anthelmintic assay are displayed in Table 2.

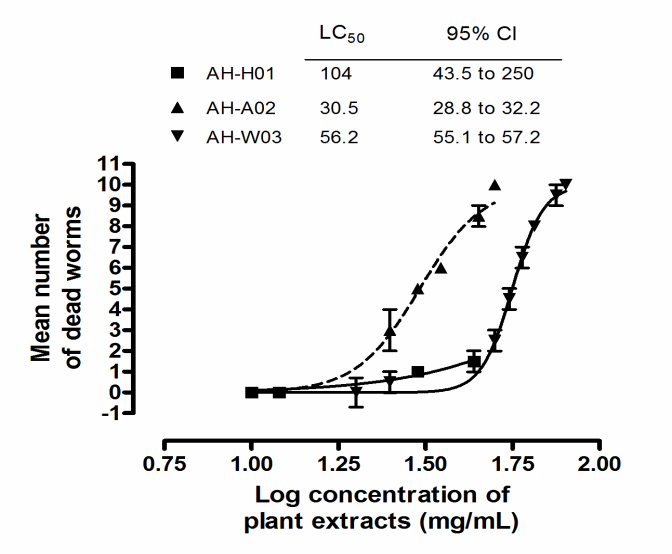

Figure 3: Concentration -response curve of adult *H. placei* worm incubated with plant extracts in normal saline (NS) incubation medium, using variable slope sigmoidal model for curve fitting. (Constraints: Bottom is constant = 0, Top is constant = 10). Sample AH-A02 is shown to be the most potent, with LC_{50} of 30.5 mg/mL determined with a narrow confidence interval (3.4 units). The excellent performance of the new method underscores the suitability of NS alone as incubation medium.

Table 2: Validation parameters for the anthelminitic activity profiling of plant extracts incubated with adult *H. placei* worm in normal saline (NS) incubation medium. Curve-fitting of worm motility test data was implemented by fitting four parameter logistic regression (variable slope) sigmoidal model

	Variable slope sigmoidal model parameters [Anthelmintic plant extracts obtained by solvents of varying polarity]		
	AH-H01	AH-A02	AH-W03
		Accuracy	
a) Best-fit values			
Bottom	0	0	0
Top	10	10	10
Log EC50	2.02	1.48	1.75
Hill Slope	1.92	4.76	9.77
EC50	104	30.5	56.2
b) Goodness-of-fit			
Degrees of freedom	6	10	16
R^2	0.835	0.964	0.984
Absolute sum of squares	0.639	4.88	4.23
Sy.x	0.326	0.699	0.514
		Reliability	
a) Standard error			
Log LC_{50}	0.155	0.0110	0.00393
Hill slope	0.684	0.640	0.932
b) 95% CI			
Log LC_{50}	1.64 to 2.40	1.46 to 1.51	1.74 to 1.76
Hill slope	0.250 to 3.60	3.34 to 6.19	7.79 to 11.7
LC_{50}	43.5 to 250	28.8 to 32.2	55.1 to 57.2
c) Constraints			
Bottom	0	0	0
Top	10	10	10

DISCUSSION

This study describes the optimization of an *in vitro* assay that is suitable for screening small molecules, phytochemicals and phytomedicines for their anthelmintic potential. In particular, assay protocols were selected by optimizing each step, such as to shorten the cycle time for a complete assay procedure. A 3-hour incubation time was found optimal, as opposed to a 6-hour incubation time. Furthermore, frequent counting of dead worms in a previous method (Sharma, et al. 1971), was also eliminated.

Levamisole is an old but still useful anthelmintic that selectively activate nematode ion channel receptors (Martin et al., 2012). It's use in combination with benzimidazoles was found to be very effective in an area where anthelmintic resistance was recorded against some other popular anthelmintics (Buttar et al., 2012). The LC_{50} obtained at the ng/mL concentration range suggested good sensitivity of *H. placei* to the drug. Levamisole hydrochloride, which was used for the investigations, is the salt form of the basic compound levamisole. The salt form has a higher water solubility profile than the free base. This enhanced water solubility explains the comparable LC_{50} obtained for the compound in both PBS-7.4 and NS incubation media. The lower solubilization capacity of PBS-7.4 did not limit the solubility of levamisole hydrochloride relative to NS. This solubility behavior of a salt will, however, not be applicable, when plant extracts are being evaluated for anthelmintic properties. The difference in solubilization capacity of PBS-7.4 and NS is expected to affect the solubility of structurally diverse phytochemicals that constitute plant extracts. This will in turn affect the concentration range that could be investigated in the assay. Incubation medium would, therefore, significantly affect the performance characteristics of the assay.

Structural diversity is the standard nature of mixture of secondary metabolites that constitute plant extracts. In order to facilitate water solubility, 30% Tween 80, a non-ionic surfactant, was used as vehicle in the preparation of stock solution of *n*-hexane extracts, due to the lipophilic nature of hexane extracts. Acetone extracts are of medium polarity, as such, 20% Tween 80 was adequate to solubilize the extract. Water extract is very soluble in water, as such, surfactant additive was unnecessary, and the stock solution was therefore prepared in normal saline. Other options, like the use of dimethylsulfoxide (DMSO) or acetone as co-solvent for stock solutions, were tried and rejected, because the co-solvents were toxic to the worms. In the anthelmintic profiling of acetone extract (AH-A02), the upper concentration limit of the solutions in PBS tested was 20 mg/mL, while the upper concentration limit of the solutions in NS tested was 50 mg/mL. The upper limit represents the maximum concentration that could possibly be prepared in each of the aqueous diluents (solution). This difference in solubilization capacity is due to variation in solvation power. Solvation power is inversely related to the amount of inorganic salts already dissolved in the aqueous solution. PBS has more salts (i.e. sodium chloride and phosphate salts) dissolved than NS, which has only sodium chloride of the same concentration as found in PBS. This higher solvation power explains the higher upper concentration limit found in NS compared with PBS.

The inorganic salts are solvated by ion-dipole interaction with water molecules, which significantly limits the dipole of water molecules available to hydrate structurally diverse molecules found in plant extracts (Chang, 2000). This explains the lower equilibrium solubility of the plant extract in PBS-7.4, which has a higher ionic strength, relative to NS. The direct consequence of a higher upper limit of concentration permissible in NS is the use of greater number of data points in the regression analysis, which in turn improves the associated estimate of uncertainty like 95% CI, thus improving the reliability of LC_{50} determination.

Acetone and aqueous extracts contain secondary metabolites that are more water soluble and hence are more soluble in the aqueous incubation medium, than constituents of *n*-hexane extract. This is attested to by the fact that the aqueous extract, AH-W03, has the highest upper limit of the concentration tested of all the 3 extracts. The acetone extract, AH-A02, however exhibited the greatest potency, as revealed by the lowest LC_{50} of 30.5 mg/mL. The most potent anthelmintic principles in the leaf of *S. mombin* were thus shown to be compounds with polar fragments rather than non-polar compounds. This assay results thus provided direction for the task of isolating new chemical entities with anthelmintic properties from the plant. Reliable ranking of relative potency of plant extracts obtained by using several solvents of varying polarities ensured structurally similar compounds with similar physicochemical properties were tested together. Overall, curve-fitting analysis of concentration-response data was shown to be a reliable method of estimating the relative potency of anthelmintics. This is consistent with the findings of other workers (Kotze, et al. 2004, Ademola et al. 2005, Ademola et al. 2007, Ademola et al. 2009).

CONCLUSION

S. mombin extracts are active against adult *H. placei* worm, suggesting broad spectrum activity of the active phytochemicals.

The optimised anthelmintic assay demonstrated excellent performance and enhanced sample throughput in screening phytomedicines for anthelmintic activity. It could find application in the ongoing effort to develop alternative anthelmintic therapy for managing helminthosis of livestock.

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