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Original Article

In vitro and *in vivo* anthelmintic response of the seeds of *Amomum subulatum* roxb and *Vitex negundo*

Atividade anti-helmíntica *in vitro* e *in vivo* das sementes de *Amomum subulatum* roxb e *Vitex negundo*

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Abstract

The current study was designed to check the anthelmintic activities of some local plants. Seeds of *Amomum (A.) subulatum* and *Vitex (V.) negundo* in different solvents were subjected to *in vitro* (adult motility assay; AMA and egg hatch assay; EHA) and *in vivo* (faecal egg count reduction test; FECRT) anthelmintic activity testing protocols using *Haemonchus (H.) contortus* as an experimental model. The results of AMA, EHA, and FECRT were statistically analysed through linear regression and Duncan multiple range test. In AMA test, at 50 mg mL^{-1} concentration, the percent mortality of *H. contortus* was higher in *A. subulatum* than *V. negundo*, whereas, in EHA test, *A. subulatum* was proven better ovicidal (LC_{50}=14.2 µg mL^{-1}) than *V. negundo* (LC_{50}= 65.7405 µg mL^{-1}). The FECRT also indicated the better efficacy of *A. subulatum* than *V. negundo* against natural infection of gastrointestinal (GI) parasites. The crude powder of plants used in this study showed 29.6% to 57.7% anthelmintic. The reduction rate was found higher for *A. subulatum* (3 g kg^{-1}) as compared to *V. negundo* (7 g kg^{-1}). Reagrding efficacy analysis of solvents used for plants extract, ethyl acetate and chloroform were found better in increasing ovicidal activity in adult worms (*in vitro* testing), whereas, the crude aqueous methanol was found better than the crude powders in *in vivo* testing. It will be beneficial to document the indigenous knowledge to standard scientific procedures for their validation. This study will help to motivate the farmers to make a better choice of cultivation of the indigenous plants because of their varying efficacies as an alternative preventive approach against the GI parasitic infections.

Keywords: *Amomum subulatum*; *Vitex negundo*; *Haemonchus contortus*; *In vivo* and *in vitro* assay.

Resumo

O presente estudo foi desenhado para verificar as propriedades anti-helmínticas de algumas plantas locais. Sementes de *Amomum (A.) subulatum* e *Vitex (V.) negundo* em diferentes solventes foram submetidas à análise de atividade anti-helmíntica *in vitro* (ensaio de motilidade de adultos; AMA e teste de eclosão de ovos; EHA) e *in vivo* (teste de redução da contagem de ovos nas fezes; TRCOF), usando o *Haemonchus (H.) contortus* como modelo experimental no protocolo de teste. Os resultados dos testes AMA, EHA e TRCOF foram analisados estatisticamente por meio de regressão linear e teste de Duncan. No teste AMA, na concentração de 50 mg mL-1, o percentual de mortalidade de *H. contortus* foi maior com o uso de *A. subulatum* do que com *V. negundo*, enquanto, no teste EHA, *A. subulatum* apresentou maior ação ovicida (LC50=14,2 µg mL- 1) do que *V. negundo* (LC50= 65,7405 µg mL-1). O

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TRCOF também indicou a melhor eficácia do uso de *A. subulatum* do que de *V. negundo* contra a infecção natural de parasitas gastrointestinais (GI). O extrato bruto seco das plantas utilizadas neste estudo apresentou 29,6% a 57,7% de atividade anti-helmíntica. A taxa de redução observada com o uso de *A. subulatum* (3 g kg-1) foi maior que com o uso de *V. negundo* (7 g kg-1). Em relação à análise da eficácia dos solventes utilizados para o extrato de plantas, o acetato de etila e o clorofórmio apresentaram maior ação ovicida em vermes adultos (testes *in vitro*), enquanto o extrato bruto metanólico aquoso apresentou maior eficácia do que os extratos brutos secos em testes *in vivo*. Consideramos vantajoso documentar o conhecimento indígena relativos aos procedimentos científicos padronizados, para sua validação. Este estudo irá servir de motivação para que os agricultores façam escolhas melhores referentes ao cultivo das plantas indígenas devido às suas diferentes eficácias comprovadas, servindo como alternativa para a abordagem preventiva contra as infecções parasitárias GI.

Palavras-chave: *Amomum subulatum*; *Vitex negro*; *Haemonchus contortus*; Ensaio *in vivo* e *in vitro*.

1. Introduction

Helminths especially gastrointestinal (GI) nematodes are highly abundant due to easy mode of transmission, wide variety of hosts and vectors, and low level of awareness which cause huge economic losses to livestock producers (Raza et al., 2014; Rizwan et al., 2017). Ethnoveterinary Medicine (EVM) has been used for many years and is widely used in many areas of the world including Indo-Pak as a traditional medicine in curing animal diseases. Use of EVM is of great significance in modern veterinary medicine, especially in sub-continent countries due to easy accessibility and low cost (Iqbal et al., 2006; Goraya et al., 2013; Hamad et al., 2014; Rizwan et al., 2019; Ahmad et al., 2020).

Anthelmintic resistance shown by nematodes against all chemical groups, and chemical residues in animals in underdeveloped countries such as Pakistan is a great problem (Muhammad et al., 2005; Iqbal et al., 2005; Jabbar et al., 2007; Hussain et al., 2008; Tabassam et al., 2008; Chirag et al., 2013). Due to this factor, scientists from developed countries have also been screening plants for their anthelmintic properties (Tabuti et al., 2003). Studies and EVM surveys have revealed effective and extensive use of some plants with anthelminthic properties (Jabbar et al., 2007; Hussain et al., 2008; Goraya et al., 2013; Badar et al., 2017).

The fruit of *Amomum (A.) subulatum*, commonly called '*Bari Ilaichi*' or '*Heel Kalan*', is a GI treatment of choice for producing carminative, digestive, antiemetic and stomachic effects (Sherpa et al., 2015). *A. subulatum* seeds are either used as flavoring spice, or as a diuretic, expectorant or cardiac tonic. The seeds of *A. subulatum* are also used to treat various ailments and conditions such as skin diseases, dyspepsia, cardiac debility, ulcers, hyperacidity, dysentery, wounds, cough, fever, gonorrhea, liver congestion (Sharma et al., 2002; Parmar et al., 2009). Moreover, the seeds of *A. subulatum* also used to treat digestive disorders, anestrous, genital prolapse, and anhidrosis in animals (Muhammad et al., 2005; Dilshad et al., 2008). For the therapy of agalactia, anorexia and worm infestation, powdered seeds of *A. subulatum* mixed with molasses, sodium chloride and ammonium chloride were found effective (Hussain et al., 2008). *Vitex (V.) negundo* (*Vitex* spp.) is used for the treatment of snakebite, asthma, depression, allergy, skin diseases, malaria, body pains, wounds and different venereal diseases (Neuwinger, 2000). For the treatment of worm infection, powdered seeds of *V. negundo* mixed with jiggery showed excellent results (Dilshad et al., 2008).

In Pakistan, several medicinal plants have been documented from various regions. People from different parts of the country are using these medicinal plants for the treatment of different diseases including parasitic diseases and transfer this knowledge from generation to generation (Goraya et al., 2013; Badar et al., 2017, 2021). Still, these plants are used by native healers and, they don't have any knowledge about the active ingredients. In the present study, we select the *A. subulatum* Roxb and *V. negundo* L plants which are being used by native healers for the treatment of parasitic infection (Badar et al., 2017). The objective of the study was to check the anthelmintic efficacy of *A. subulatum* Roxb and *V. negundo* L through *in vitro* and *in vivo* assays against *Haemonchus contortus* models.

2. Materials and Methods

2.1. Selection and procurement of plants

The two seed plants viz; *A. subulatum* Roxb (family Zingiberaceae) and *V. negundo* L. (family Verbenaceae) were selected during a documentation survey of the herbs as candidates to treat the animals for helminths infection (Badar et al., 2017). The selected plants' seeds were purchased from a local market and also directly harvested from the fields.

2.2. Extraction and fractionation of seeds

2.2.1. Extraction

Grinding of seeds was done after drying, and crude aqueous-methanol extracts (CAME) were prepared following the guidelines described by Tabassam et al. (2008). Ground powder was soaked in an aqueous solvent (aqueous-methanol 30:70) for three days and passed through muslin cloth and filter paper. The whole procedure was repeated thrice. Afterwards, the combined filtrate was evaporated to get CAME using rotary evaporator at 40°C at low pressure.

2.2.2. Fractionation

Crude extract was further processed for fraction with organic solvents e.g., ethyl acetate, petroleum spirit and

chloroform. All the crude extracts were fractionated by using organic solvents such as chloroform, petroleum, ethyl acetate and spirit (Williamson et al., 1998). Distill water (20 mL) was used to dissolve 20 g of crude extract in separating funnel. In the next step, the petroleum spirit (60 mL) was added to the funnel followed by vigorous shaking and kept undisturbed for 30 minutes for the separation phase. Petroleum layer containing soluble part of the extract was separated and petroleum ether (60 mL) was added followed by vigorous shaking and separation phase process until getting a clear layer of petroleum spirit. The solvent was evaporated using a rotary evaporator to get spirit petroleum fractions. The ethyl acetate was used to fractionalize the remaining part of the extract following the same procedure as described above.

2.3. Parasitological procedures

2.3.1. Adult Motility Assay (AMA)

The efficacy of different fractions and the crude extracts was accessed by taking *Haemonchus (H.) contortus* (live) from abomasum of slaughtered sheep as described by Singh et al. (1985). The worms were separated in petri dishes (minimum 10 worms in each) and exposed to CAME, ethyl acetate fraction, petroleum spirit fraction and chloroform fraction at 1.56, 3.12, 6.25, 12.5, 25 and 50 mg mL^{-1} concentrations. Levamisole at 0.55 mg mL^{-1} and phosphate buffer saline (PBS) were used as a positive and negative controls, respectively. All treatments were conducted in three replicates at room temperature. Observation made for the accuracy of experiment was motility inhibition at 0, 2, 4, 6, 8, 10, 12 and 24 hours intervals. For each treatment, the number of survived and dead worms was noted.

2.4. Egg Hatch Assay (EHA)

The CAME, ethyl acetate, petroleum spirit and chloroformfractions of crude extract were used to check anthelmintic activity keeping Albendazole as a positive control. Briefly, 24 multiwell plates were used for each solvent with 5 dilutions i.e., 12, 1.2, 0.12, 0.012 and 0.0012 mg mL^{-1} in triplicate. 250 eggs were in 1.5 mL of water were poured in each well. Following 36 hours incubation of plates at 28°C, eggs percent hatching (live or dead larvae) was counted using an inverted microscope.

2.5. Faecal Egg Count Reduction Test (FECRT)

At day 0, 14 different groups of sheep were formed which are one to four months in age and were previously infected with *H. contortus*. Group was selected using a complete randomization method based on their live weights. Layout plan for crude powder and CAME of Seeds of *A. subulatum* and *V. negundo* given to different groups of sheep naturally infected with *H. controtus* is given in Table 1. The faecal egg counts were done on days 0, 4, 8 and 12 post-treatments from each animal as given by Badar et al. (2021).

2.6. Statistical analyses

All measurements were summarized as Mean ± SE. Adult mortality was determined by Abbott's formula and probit equation was used to determine the association of Probit of kill and log concentration of treatment (Abbott, 1925). For egg hatch test, linear regression was used to determine the lethal concentration 50 (LC_{50}). Duncan multiple range (DMR) test was used to compare eggs per gram (Mean+SEM) of faeces.

Table 1. Layout plan for crude powder and crude aqueous methanol extract of seeds of *Amomum subulatum* and *Vitex negundo* given to different groups of sheep naturally infected with *Haeminchus controtus*.

Group	Treatment	Dose rate
1	untreated or control	
2	levamisole	7.5 mg per kg b.wt.
3	crude powder (CP) of *A. subulatum* (S.)	0.5 g per kg b.wt.
4	crude powder (CP) of *A. subulatum* (S.)	1 g per kg b.wt.
5	crude powder (CP) of *A. subulatum* (S.)	3 g per kg b.wt.
6	crude powder (CP) of *V. negundo* (S.)	1 g per kg b.wt.
7	crude powder (CP) of V. negundo (S.)	4 g per kg b.wt.
8	crude powder (CP) of V. negundo (S.)	7 g per kg b.wt.
9	*CAME of *A. subulatum* (S.)	0.5 g per kg b.wt.
10	CAME of *A. subulatum* (S.)	1 g per kg b.wt.
11	CAME of *A. subulatum* (S.)	3 g per kg b.wt.
12	CAME of *V. negundo* (S.)	1 g per kg b.wt.
13	CAME of V. negundo (S.)	4 g per kg b.wt.
14	CAME of V. negundo (S.)	7 g per kg b.wt.

*CAME = Crude aqueous methanol extract.

3. Results

3.1. In vitro anthelmintic activity

In AMA, after 10 hours post-exposure of *H. contortus* with 50 mg mL^{-1} of *A. subulatum*, 73.3% mortality was reported. The reference drug (Levamisole) revealed 100% worm mortality within 2 hours post-exposure. Among the fractions of *A. subulatum*, ethyl acetate produced the most effective anthelmintic activity. However, *V. negundo* extract in ethyl acetate did not show up to the mark anthelmintic activity. The CAME, chloroform and petroleum spirit fractions of both plants were found least responsive. The results of AMA for various treatments of fractions of *A. subulatum* and *V. negundo* are summarized in Table 2.

In EHA, the CAME of *A. subulatum* was considered to be better ovicidal based on its lower LC_{50} (14.1773 µg mL^{-1}) than that of *V. negundo*. The eggs exposed to aqueous fraction of *V. negundo* and chloroform fraction of *A. subulatum* showed more pronounced response, whereas, petroleum spirit fraction of both plants' extract was effective in dose-dependent manner (Table 3).

Table 2. Comprative efficacy of crude aqueous methanol extracts, chloroform fractions, ethyl acetate fractions, petroleum spirit fractions and aqueous fractions of *Amomum subulatum* and *Vitex negundo* with Levamisole on the survival of *Haeminchus contortus* of sheep.

Treatments mg mL^{-1}	***Amomum subulatum* (Seeds)**				***Vitex negundo* (Seeds)**			
	Mean±SE of dead worms at different hours				**Mean±SE of dead worms at different hours**			
Hours post-exposure	**0 hour**	**2 hours**	**6 hours**	**10 hours**	**0 hour**	**2 hours**	**6 hours**	**10 hours**
Levamisole 0.5	0^{r}	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}	0^{k}	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}
PBS	0^{r}	0^{r}	0^{r}	0^{r}	0^{k}	0^{k}	0^{k}	0^{k}
Crude Aqueous Methanolic Extract								
1.56	0^{m}	0^{m}	0.33 ± 0.33^{lm}	1.00 ± 0.00^{klm}	0^{o}	0^{o}	0^{k}	0.67 ± 0.67^{mno}
3.12	0^{m}	0^{m}	1.00 ± 0.00^{klm}	1.67 ± 0.00^{ijk}	0^{o}	0^{o}	0.33 ± 0.33^{ijk}	1.00 ± 0.58^{lo}
6.25	0^{m}	0^{m}	1.33 ± 0.33^{jkl}	2.33 ± 0.33^{hij}	0^{o}	0^{o}	0.67 ± 0.33^{hk}	3.00 ± 0.58^{fi}
12.5	0^{m}	0^{m}	1.67 ± 0.33^{ijk}	3.33 ± 0.33^{h}	0^{o}	0^{o}	1.67 ± 0.33^{gj}	4.00 ± 0.58^{ef}
25	0^{m}	1.67 ± 0.33^{ijk}	3.00 ± 0.58^{h}	6.00 ± 0.58^{def}	0^{o}	1.00 ± 0.58^{lo}	2.33 ± 0.33^{eh}	4.33 ± 0.33^{de}
50	0^{m}	2.67 ± 0.88^{hi}	5.67 ± 0.33^{efg}	7.33 ± 0.67^{bc}	0^{o}	2.00 ± 0.58^{il}	4.00 ± 0.58^{cde}	7.00 ± 0.58^{c}
Chloroform fractions								
1.56	0^{r}	0^{r}	0.67 ± 0.67^{qr}	1.67 ± 0.33^{nq}	0^{k}	0^{k}	0^{k}	0.33 ± 0.33^{jk}
3.12	0^{r}	0^{r}	1.33 ± 0.33^{or}	2.67 ± 0.33^{ko}	0^{k}	0^{k}	0.67 ± 0.33^{ijk}	1.33 ± 0.33^{gj}
6.25	0^{r}	0.67 ± 0.33^{qr}	2.00 ± 0.00^{mq}	3.00 ± 0.00^{jn}	0^{k}	0^{k}	1.00 ± 0.00^{hk}	1.67 ± 0.33^{fi}
12.5	0^{r}	1.33 ± 0.33^{or}	2.67 ± 0.67^{ko}	4.00 ± 0.58^{gk}	0^{k}	0^{k}	1.33 ± 0.33^{gj}	2.33 ± 0.33^{dg}
25	0^{r}	1.67 ± 0.33^{nq}	4.33 ± 0.67^{fj}	5.33 ± 0.67^{efg}	0^{k}	0.33 ± 033^{jk}	2.00 ± 0.58^{eh}	3.00 ± 0.58^{cde}
50	0^{r}	3.33 ± 0.88^{im}	5.67 ± 0.88^{ef}	7.67 ± 0.33^{bc}	0^{k}	1.00 ± 0.58^{hk}	3.00 ± 0.58^{cde}	4.67 ± 0.67^{b}
Ethyl acetate fractions								
1.56	0^{r}	0^{r}	1.33 ± 0.33^{opq}	2.67 ± 0.33^{kn}	0^{m}	0^{m}	0^{m}	0.33 ± 0.33^{lm}
3.12	0^{r}	0.67 ± 0.33^{qr}	2.33 ± 0.33^{lo}	3.33 ± 0.67^{jkl}	0^{m}	0^{m}	0.67 ± 0.33^{klm}	1.33 ± 0.33^{ijk}
6.25	0^{r}	1.00 ± 0.00^{pqr}	2.67 ± 0.33^{kn}	4.33 ± 0.88^{hij}	0^{m}	0^{m}	1.33 ± 0.33^{ijk}	2.00 ± 0.00^{ghi}
12.5	0^{r}	1.67 ± 0.33^{nq}	4.33 ± 0.33^{hij}	6.00 ± 0.58^{ef}	0^{m}	0^{m}	1.67 ± 0.33^{hij}	2.33 ± 0.33^{gh}
25	0^{r}	2.00 ± 0.58^{mp}	5.33 ± 0.33^{fgh}	9.00 ± 0.58^{ab}	0^{m}	0.67 ± 0.33^{klm}	2.00 ± 0.00^{ghi}	4.00 ± 0.58^{de}
50	0^{r}	4.33 ± 0.33^{hij}	7.00 ± 0.58^{de}	10.00 ± 0.00^{a}	0^{m}	1.67 ± 0.33^{hij}	3.33 ± 0.88^{ef}	6.00 ± 0.58^{b}
Petroleum spirit fractions								
1.56	0^{n}	0^{n}	0^{n}	1.00 ± 0.00^{klm}	0^{k}	0^{k}	0^{k}	0.33 ± 0.33^{jk}
3.12	0^{n}	0^{n}	0^{n}	1.67 ± 0.33^{ijk}	0^{k}	0^{k}	0^{k}	0.67 ± 0.33^{ijk}
6.25	0^{n}	0^{n}	0.67 ± 0.33^{lmn}	2.33 ± 0.33^{ghi}	0^{k}	0^{k}	0^{k}	1.33 ± 0.33^{ghi}
12.5	0^{n}	0^{n}	1.33 ± 0.33^{jkl}	2.67 ± 0.33^{fgh}	0^{k}	0^{k}	0.67 ± 0.33^{ijk}	2.00 ± 0.58^{efg}

Motility of worms were observed for 24 hours at every 2 hours interval. However, data of 0, 2, 6 and 10 hours has been given which deemed enough to meet the objectives of the experiment. Values having common alphabet superscripts indicate non-significant results at 95% confidence interval.

Table 2. Continued...

Treatments mg mL^{-1}	*Amomum subulatum* (Seeds)				*Vitex negundo* (Seeds)			
	Mean±SE of dead worms at different hours				Mean±SE of dead worms at different hours			
Hours post-exposure	0 hour	2 hours	6 hours	10 hours	0 hour	2 hours	6 hours	10 hours
25	0^{n}	0^{n}	2.00 ± 0.58^{hij}	3.33 ± 0.33^{def}	0^{k}	0^{k}	1.33 ± 0.33^{ghi}	2.67 ± 0.33^{de}
50	0^{n}	0^{n}	3.00 ± 0.58^{efg}	4.67 ± 0.67^{bc}	0^{k}	0^{k}	2.67 ± 0.67^{de}	4.33 ± 0.33^{b}
Aqueous fractions								
1.56	0^{k}	0^{k}	0^{k}	0.33 ± 0.33^{jk}	0^{l}	0^{l}	0^{l}	0.33 ± 0.33^{kl}
3.12	0^{k}	0^{k}	0^{k}	0.67 ± 0.33^{ijk}	0^{l}	0^{l}	0^{l}	1.33 ± 0.33^{ijk}
6.25	0^{k}	0^{k}	0^{k}	1.33 ± 0.33^{ghi}	0^{l}	0^{l}	0.67 ± 0.33^{jkl}	1.67 ± 0.33^{hij}
12.5	0^{k}	0^{k}	0^{k}	2.33 ± 0.33^{ef}	0^{l}	0^{l}	1.00 ± 0.58^{ijkl}	2.67 ± 0.67^{fgh}
25	0^{k}	0^{k}	1.33 ± 0.33^{ghi}	2.67 ± 0.33^{de}	0^{l}	0.33 ± 0.33^{kl}	1.67 ± 0.33^{hij}	3.67 ± 0.88^{ef}
50	0^{k}	0^{k}	2.33 ± 0.33^{ef}	5.00 ± 0.58^{b}	0^{l}	0.67 ± 0.67^{jkl}	3.00 ± 0.58^{fg}	5.67 ± 0.67^{bc}

Motility of worms were observed for 24 hours at every 2 hours interval. However, data of 0, 2, 6 and 10 hours has been given which deemed enough to meet the objectives of the experiment. Values having common alphabet superscripts indicate non-significant results at 95% confidence interval.

Table 3. *In vitro* effect of seeds of *Vitex negundo* and *Amomum subulatum* and LC_{50} of the different fractions and crude aqueous methanol extracts on hatching (%) of *Haemonchus contortus* eggs.

Treatments	fraction concentrations mg mL^{-1}					LC_{50} µg mL^{-1}
	1.2	12	120	1200	12000	
Albendazole	79	64	40	5	0	0.0345
***Amomum subulatum* (Seeds)**						
CAME	41	39	28	19	6	14.1773
Choloroform fraction	38	37	33	27	13	19.1514
Ethyl acetate fraction	45	37	28	17	4	19.6203
Aqueous fraction	44	42	33	23	17	21.4279
Petroleum spirit fraction	45	37	33	27	13	254.9207
***Vitex negundo* (Seeds)**						
CAME	77	66	46	31	12	65.7405
Aqueous fraction	73	61	40	34	14	39.7628
Petroleum spirit fraction	75	58	48	36	18	62.5137
Ethyl acetate fraction	77	66	50	39	17	93.3188
Choloroform fraction	89	67	49	36	26	192.0218

3.2. In vivo anthelmintic activity

The anthelmintic and dose dependant efficacies of crude powder (CP) and methanol extract of both plants were studied. Efficacy of CP and methanol extracts of study plants based on FECRT at days 0, 4, 8 and 12 post-treatment is shown in Figure 1. Anthelmintic efficacy was recorded from 29.6% to 57.5%. Using *A. subulatum* at the rate of 3 g kg^{-1} showed a higher reduction (57.5%) in faecal egg counts compared to *V. negundo* which was 8 g kg^{-1}. The CP and methanol extracts showed different anthelmintic efficacies. The effects of both plants' treatments were statistically significant ($P<0.05$) from the untreated control. The variations in the egg per gram (EPG) of the Levamisole treated groups with those treated with plant extracts were statistically different ($P<0.05$).

4. Discussion

There is increasing evidence in support of the hypothesis that bioactive secondary phytochemicals are relatively abundant and are likely to initiate drug discovery. Due to the difference in their polarities, these phytochemicals have variable affinities for different solvents. Different chemical compounds are present in each fraction according to the solvents used. Active compounds present in the fractions are the main responsible agents for biological activities. So, there is a need to check the efficacies of fractions of different botanical compounds to be used as anthelmintics (Hamad et al., 2014).

Phytochemical studies of *A. subulatum* fruits have unveiled essential oils (Lawrence, 1970), anthocyanins

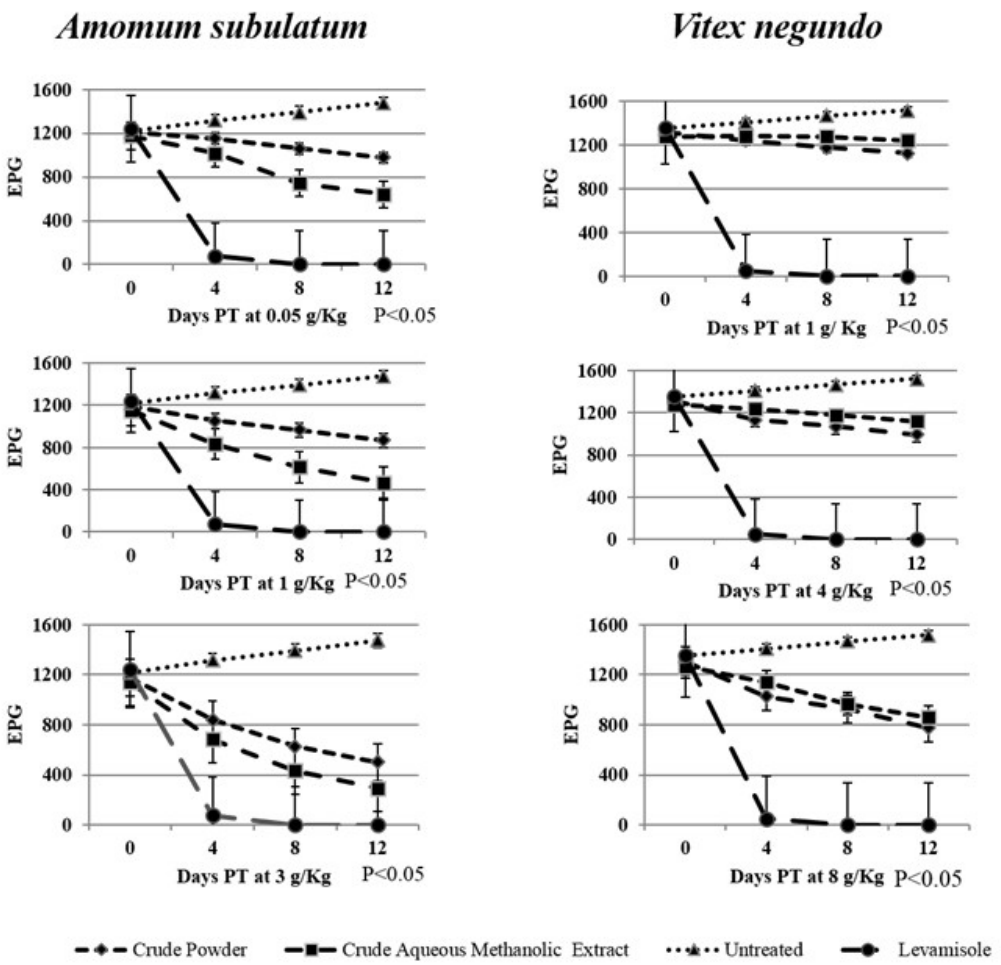

Figure 1. Effects of various solvents extracts of *Vitex negundo* and *Amomum subulatum* on naturally infected mixed species of gastrointestinal nematodes' eggs in sheep feces. Eggs per gram (EPG) is the measure of efficacy of extract present on y-axis whereas, day in which effect comes out along with dose is mentioned on x-axis.

(Lakshmi and Chauhan, 1976), a flavanone, chalcone (Rao et al., 1976) and aurone (Lakshmi and Chauhan, 1977). Lawrence (1970) has reported 1, 8-cineol (more than 70%) dominating 3% essential oil and varying amounts of terpinyl acetate, limonene, terpineol, terpinene, and sabinene from *A. subulatum* seeds. The essential oil extracted from *A. subulatum* fruits possess antimycotic activity (Mishra and Dubey, 1990). Phytoconstituents of *A. subulatum* like volatile oils, glycosides, flavonoids and terpenoids (Defeudis et al., 2003) are being used due to their hepatoprotective and antioxidant activities (Jafri et al., 2001). The gastroprotective effect of *A. subulatum* is attributed to petroleum ether fractions and essential oil that decreases gastric motility (Bonjar, 2004). Rao et al. (1976) reported gastroprotective activity of *A. subulatum*'s phenolic fractions i.e., flavanones, anthocyanins or aurones. Bonjar (2004) reported the antibacterial activity of *A. subulatum*. Kumar (2016) determined the aqueous and ethanolic extracts of *A. subulatum* Roxb. rhizomes. The concentration-dependent anthelmintic results showed that only 100 mg/mL ethanolic extracts of *A. subulatum* were effective against parasites. He reported that the anthelmintic activity of the ethanolic extracts was much higher than the aqueous extracts. In a study conducted by Alam et al. (2020), all the doses of ethanolic extract of *A. subulatum* showed dose dependent anthelmintic activity similar to our study.

The genus *Vitex* is supposed to have hormone-like properties. *Vitex megapotamica* is found to be a source of 20-hydroxy ecdysone which is known as an insect molting hormone (Subramanian and Misra, 1979). The antiarthritic effect was reported from the leaves of plants of genus *Vitex* (Chaturvedi and Singh, 1965). Subramanian and Misra (1979) reported two new flavonoid glycosides from the stem bark of *V. negundo*. Hypoglycemic, anti-inflammatory, hypouricaemic and analgesic effects have been reported from *V. negundo* extracts (Umamaheswari et al., 2007). Flavonoid-rich fractions of its seeds have anti-oxidant and antiandrogenic properties (Das et al., 2004). *Vitex negundo* has also been used as hepato-preventive, anti-histaminic

and CNS depressant (Gupta et al., 1999). Zhong et al. (1996) reported significant analgesic activity of aqueous extract from the seeds of *V. negundo*.

The change in anthelmintic efficacies of different fractions might be due to the change in parasitic targets and plant chemistry. Several tropical legumes such as *Lespedeza* (*L.*) *cuneata* (50 g CTs kg^{-1}) has also shown some promising results in reducing total faecal egg output, faecal egg counts (FECs) by 57–100%, and the number of nematodes (*Teladorsagia* sp., *Trichostrongylus* and *Haemonchus*) in goats (Min and Hart, 2003). A higher level of dried *Acacia karoo* leaves can significantly reduce FECs and *H. contortus* load in goats (Kahiya et al., 2003). The anthelmintic targets may vary with the parasitic stages. O'Grady and Kotze (2004) have discussed that mechanism-based assays for anthelmintic effects showed a potential problem with the whole-organism screening. These assays can be applied easily to the free-living parasitic stages such as larval development, larval motility assays and egg hatch, while the eventual use of the anthelmintics will be directed to the host dependant parasitic stages. In an *in vitro* assay, 0.16 mg/mL inhibitory concentration (IC50) of ethyl acetate extract of *V. negundo* leaves was recorded (Sahare and Singh, 2013). Ethanol extract of *V. negundo* leaves at 50 mg/mL requires less time to cause paralysis and death of parasites as compared to ethanol extract of roots of the *Tamarindus (T.) indica* (Raul et al., 2012).

Synthetic anthelmintic drugs show unwanted effects on the animal body like urticaria, gastrointestinal disturbance, bronchospasm, dizziness, in coordination, paraesthesias, and vertigo. In pregnanet animals those with compromised hepatic and renal function these drugs are contraindicated. The formulation of anthelmintic drugs from *A. subulatum* and *V. negundo* will be safe, cost effective, natural and probably will not show any such unwanted effects. Still there is need to determine the main active ingridients from these plants which are responsible for the anthelmintic activity.

5. Conclusions

The phytotherapeutic approaches have been found promising *in vitro* and *in vivo* for the remedies of the GI parasites in general and *H. contortus* in specific. It is recommended to document the indigenous knowledge after subjected to standard scientific procedures for their validation. Both the study plants (*A. subulatum* and *V. negundo*) showed anthelmintic activity comparable to the standard therapy, however, among various fractions, ethyl acetate fractions at 50 mg mL^{-1} of *A. subulatum* and CAME at 50 mg mL^{-1} of *V. negundo* were found the most effective against *H. contortus in vitro*. *In vivo* trials indicated the CAME at 3 g kg^{-1} of *A. subulatum* and crude powder at 8 g kg^{-1} of *V. negundo* as the most promising dose. The study plants may initially be recommended as a short-term prophylactic approach to cultivate in the grazing area which may reduce the severity of GI parasitism in the livestock population.

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