Punjab University Journal of Zoology

OPEN ACCESS

38(2): 221-228 (2023)
https://dx.doi.org/10.17582/journal.pujz/2023.38.2.221.228

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Research Article

Comparative Efficacy of Neem (*Azadirachta indica*), Levamisole and Combination of Levamisole, Oxyclozanide and Cobalt against Gastrointestinal Parasites: A Way Forward for Public (One) Health

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Article History
Received: October 10, 2023
Revised: November 12, 2023
Accepted: November 30, 2023
Published: December 12, 2023

Authors' Contributions
MAR, AZD, MMA and TU presented the concept. HH, KHA, STM and NR planned methodology. MWI, NR, MAR, TU, KHA amd AZD wrote and edited the original draft. MAR and AZD administered project. AZD supervised the project. TU, NR, BB, KHA, MMA, MAR and STM performed formal analysis. MAR, MMA and TU did funding acquisition and resources. MAR, KHA, HH and TU did investigation. MMA, BB, STM and MWI did validation

Keywords
One health, Public health, Traditional Medicine, Drugs comparison, GINs

Abstract | The article illustrates the *in vivo* anthelmintic activity of seeds of *Azadirachta indica* and other synthetic drugs comparatively compare and to justify the said seed consumption as an anthelmintic by the customary therapists of animals in South-Asia. Crude aqueous extract (CAE) from seeds of *A. indica* at the dosage of 2 g/kg of body weight, levamisole HCL at the dose rate of 7.5 mg/kg and 3.0% oxyclozanide BP (vet), 1.5 levamisole hydrochloride BP (vet) and 0.382% cobalt sulphate in combination were administered orally to the cattle naturally infected with mixed species of gastrointestinal nematodes. The study design also included infected but un-medicated and uninfected and un-medicated controls. Faecal egg count reduction and larval counts from *coprocultures* were carried out pre and post treatments to analyze the anthelmintic activity. There was significant difference among the various treatment ($p< 0.05$) and on various days ($p<0.05$). The highest decrease in the egg per gram was depicted by levamisole; the reduction in EPG was 99.05% as compared to day zero (pre treatment), the lowest reduction of fecal egg per gram was revealed by CAE of neem, the reduction in eggs per gram was 11.25% as compare with day zero and levamisole, oxyclozanide and cobalt in combination represented 98.8% reduction in faecal egg count on day 21. The average decrease in mean larval count was 12.8, 97.97 and 95.9% by neem, levamisole and combination of levamisole, oxyclozanide and cobalt respectively on day 21. As a whole, the highest effect or highest reduction in mean larval count was delineated by group levamisole on day 21.

Novelty Statement | *In vivo* anthelmintic activity of *Azadirachta indica* seeds (crude aqueous extract) against gastrointestinal nematodes of cattle (eggs and larvae along with identification) pertaining to Public (One) Health has categorically been studied for the first time in Lahore with a proper treatment protocol and comparison with levamisole, oxyclozanide and the combination of both drugs.

To cite this article: Raza, M.A., Durrani, A.Z., Ali, M.M., Usman, T., Bano, B., Rubab, N., Mehdi, S.T., Iqbal, M.W., Akhtar, K.H. and Hameed, H., 2023. Comparative efficacy of neem (*Azadirachta indica*), levamisole and combination of levamisole, oxyclozanide and cobalt against gastrointestinal parasites: A way forward for public (One) health. *Punjab Univ. J. Zool.*, **38**(2): 221-228. https://dx.doi.org/10.17582/journal.pujz/2023.38.2.221.228

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Introduction

The livestock sector is one of the important economic sources in many countries particularly in rising regions (Al-Rofaai *et al.*, 2012). In order to trounce this issue of great magnitude, the livestock sector is there which has come up in a very remarkable way in previous 40 years and is doing its maximum performance. Parasitic nematodes are very much prevalent and they cause reasonably significant infectious diseases in grazing livestock, especially in ruminants in the tropics and subtropics (Githiori *et al.*, 2003). Various types of analyses of plants including phytochemical analysis present how to check the gastrointestinal (GIT) parasites. This natural approach or herbal medicines can offer alternative methods for effective and economical control of parasitic diseases (Chagas *et al.*, 2008).

The oil of neem and other preparations have been used in the treatment of various disorders and diseases (Vinod *et al.*, 2011). The surfacing of anthelmintic confrontation or resistance in production systems of grazing animals is considered an inspiration for the discovering new methods to control the parasites of GIT. Responsiveness of the topic has also come into view from several national and international conferences and workshops held in dealing with anthelmintic resistance and recent approaches (Torres-Acosta *et al.*, 2012). Most of the nematodes are subclinical, in cattle, the genera *Ostertagia* and *Cooperia* are associated with sub-optimal performance whereas most often *Teladorsagia* and *Trichostrongylus* spp. cause subclinical losses in sheep and goats. In some other regions, at certain times, species like *Nematodirus* and *Haemonchus* also cause diseases and disorders in small ruminants like sheep and goats (Kenyon and Jackson, 2012).

Neem (locally) or Azadirachta indica is extensively used in the villages of south and central Punjab including the periphery of Lahore district as part of traditional medicine system (Iqbal *et al.*, 2010).

Materials and Methods

Experimental animals

In the current experimental design, the total number of cattle was 25, aged between 2.5 to 5 years. Those animals were selected on random basis from a local herd of more than 100 animals being maintained at a Dairy Farm in the premises of Lahore (Punjab, Pakistan).

Experimental design and anthelmintic treatment

The cattle were kept in tie stalls and were offered green feed, concentrate and roughages. They were provided with water *ad-libitum*. The animals were the vaccinated regularly according to vaccination schedule in Pakistan.

Grouping of animals

The cattle were divided into five groups, the groups were 1, 2, 3, 4 and 5 comprising five (05) animals in each group and those were randomly assigned to a variety of treatment protocols as described above.

Medicinal plant

2000 grams of dried seeds of "Neem" were be purchased from the local market of Lahore, Pakistan. By using an electric grinder, the dried seeds were crushed to powder. The powder was stored in the plastic jar at 4 °C in a refrigerator until use.

Extract preparation

The preparation of crude aqueous extract (CAE) was conducted at the laboratory of the Department of Veterinary Medicine, UVAS, Lahore. "Neem" (*A. indica*) is known to be soluble in water (1.29 g l^{-1}) (Iqbal *et al.*, 2010). It has been studied by scholars that village-based use of the seeds of this plant is quite effective and this is of used as crude powder most oftenly or as water decoction against parasitic gastroenteritis (Iqbal *et al.*, 2010). The CAE of the dried seeds of "Neem" (*A. indica*) was prepared according to the standard technique (Iqbal *et al.*, 2010). In a nut shell, the seeds were ground to powdered material (200 g) and it was mixed with 1000 ml of distilled water in a flask of 1.5 liter and boiled for ninety minutes and similar method was repeated for the preparation of the remaining 1800 grams of powder. It was given ample time to cool to 40 °C. Later on, it was filtered with Whatman filter paper number 1. The CAE of "Neem" (*A. indica*) was stored at 4°C in the refrigerator until required. The yield of extract was 15.0% (w/w).

Pre-treatment infection confirmation

The infected animals (cattle) were confirmed by taking sample of each individual animal before the beginning of the current study. For this purpose, the faecal samples were collected from the animals through their rectum. The number of nematode eggs therein was determined by the floatation method. To confirm the composition of nematod species; the fecal or stool culture was performed for the identification of larvae of various species by using the standard description.

Treatment protocol

A complete randomized block design was used for studies and each of the groups was given a single dose of the drug. The cattle of group1 received CAE of "Neem" (*Azadiracta indica*) 2 g/kg through oral route. The cattle of group 2 were given 7.5 mg/kg of levamisole HCl of a multinational brand (1.5%, w/v) through oral route. The cattle of group 3 were offered 3.0% oxyclozanide BP (vet), 1.5 levamisole hydrochloride B.P (vet) and 0.382% cobalt sulphate in combination. They were given at dosage rate of 7mg/kg via oral route. The cattle of group 4 served

as positive control having infected but un-medicated and healthy cattle. The group 4 was offered Bio plex multivitamin of a well reputed Pakistani brand at the rate of 2-3 gram per animal per day in feed to boost the immune system against the parasites on compassionate grounds. The positive control group was not treated for only 21 days during the sample collection for studies. The cattle of group 5, the last group served as negative control having uninfected and un-medicated and healthy cattle. After treatment, each cattle was kept separately so that there would not be any physical contact between them. The animals were kept on cemented floor and were fed with grass as green fodder, 'wanda' as a concentrate and fresh water ad libitum as daily ration.

Samples collection and examination

Faecal samples were collected by rectal collection method by using poly-thane gloves in plastic zipper bags. Early morning, the faecal samples of each group were collected for pretreatment infection confirmation purpose to assign the animals into various groups for studies. Then after dividing the animals into groups as mentioned above starting from day 0 pre-treatment and at days 3, 7, 14 and 21 post-treatment and were collected in the morning and evaluated for the presence of eggs of worms by salt floatation technique. The eggs were counted by the McMaster procedure. The faecal culture was performed to detect larvae of different parasites of GIT. The samples of the control negative group were collected on daily basis and then direct smear and EPG were performed on a daily basis till the last day of sample collection or the 21st day.

Statistical analyses

The faecal egg count and larvae species were presented as mean ± standard error of mean described by Coles. Data obtained was subject to analysis by using two-way ANOVA and Duncan's test through SPSS13.0.0.240 software.

Results and Discussion

The following parameters were studied during the research trial.

1. Pre-treatment infection confirmation
2. Clinical findings
3. Reduction in faecal egg count
4. Faecal culture
5. Percentage of faecal larvae of nematodes of GIT on various days and its average

Pre-treatment infection confirmation

A total of 25 cattle aged between 2.5 years to 5 years were selected from a herd of more than 100 animals on a random basis from a dairy farm situated at Kot Noor Shah Pur, Near Faiz Pur inter-change, Lahore. The infected animals were confirmed before the beginning of the current study's data collection. For nematode species composition, faecal or stool culture was performed for identification of larvae using the standard description. The pre-treatment faecal examination revealed some nematodes in GIT of cattle.

Clinical findings

The naturally challenged cattle of 4 groups i.e., 1, 2, 3 and 4 exhibited clinical signs while the cattle of group 5 didn't show any kind of clinical sign of the disease. The cattle challenged with nematodes of GIT were showing signs of dullness, depression, listlessness having rough body coat, weakness, lethargy and were anorectic. They demonstrated less feed intake and the cattle were showing dizziness, fever and digestive disturbance were observed in the infected cattle. The cattle showed loose diarrhea and later on weight loss due to in-appetite (Table 1).

Table 1: Treatment protocol.

Group	Treatment	Remarks
Group 1	Crude aqueous extract of powder of seeds of Neem (*Azadirachta indica*) via oral route 15% w/v	The cattle naturally acquired infection of Nematodes of GIT.
Group 2	Levamisole HCl 7.5 mg/kg of body weight	The cattle naturally acquired infection of Nematodes of GIT.
Group 3	3.0% Oxyclozanide BP (vet), 1.5 Levamisole Hydrochloride BP (vet) and 0.382% Cobalt Sulphate in combination @7mg/kg via oral route.	The cattle naturally acquired infection of Nematodes of GIT.
Group 4	Not treated because it was a positive control group	The cattle naturally acquired infection of Nematodes of GIT.
Group 5	Not treated because it was negative control group	The cattle did not naturally acquired infection of Nematodes of GIT.

Faecal egg count

The faecal sample processing of each infected cattle for eggs per gram was carried out on days 0, 3, 7, 14 and 21. There was a gradual reduction in the faecal egg count per gram in all the positive cases as under (Table 2):

- The average eggs per gram or abbreviated as the EPG of faecal samples of group 1 on days 0, 3, 7, 14 and 21 were 800, 790, 770, 750 and 710, respectively.
- The average eggs per gram of faeces of group 2 on days 0, 3, 7, 14 and 21 were 850, 200, 50, 9 and 8, respectively.
- The average eggs per gram of faeces of group 3 on days 0, 3, 7, 14 and 21 were 750, 250, 70, 12 and 9, respectively.
- Group 4 remained as control positive and no drug was given to the animals of group 4. The mean eggs per gram of faeces of group E on days 0, 3, 7, 14 and

21 were nil because the cattle of this group were neither infected nor treated.

Table 2: Eggs per gram (EPG) on various days of various groups.

Days	Group 1 (Neem CAE)	Group 2 (levamisole)	Group 3 (combination of LEV, OXY and Co)
0	800	850	750
3	790	200	250
7	770	50	70
14	750	9	12
21	710	8	9

The highest decrease in the egg per gram was seen in group 2(8 eggs per gram of faeces, 842 eggs were reduced out of 850) on day 21 of treatment. The reduction in eggs per gram was 99.05% as compared with day zero (pretreatment). The lowest reduction of faecal egg per gram was seen in group 1 (710 eggs per gram of faeces, only 90 eggs were reduced out of 800) on day 21 of treatment. The reduction in eggs per gram was 11.25% as compared with day zero and group 3 depicted 98.8% reduction in faecal egg count on day 21 (Figure 1).

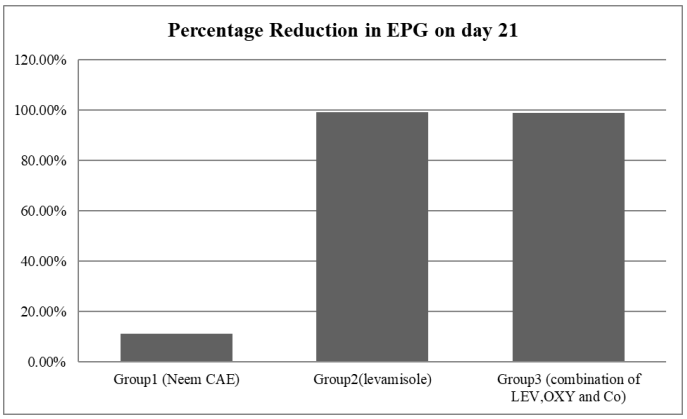

Figure 1: Graph showing decrease in faecal egg count of treated groups at day 21 post treatment comparatively by various groups.

The significant difference or $p<0.05$ illustrates that a significant difference is present and all the drugs do not have similar efficacy or on all the days there is not similar efficacy of drugs, as mentioned in the above table that p-value is 0.002 for three different treatment groups which means it is less than 0.05, ultimately there exists a significant difference between the efficacies of three groups of drugs used for the treatment of animals. Similarly for different days of treatment there is a significant difference in reduction of faecal eggs per gram of faeces. The value for days is mentioned 0.027 in the above table which shows it is less than 0.05 (α) ($p<0.05$), hence there is significant difference in efficacy on various days. There is neither a non-significant difference among three groups of treatment nor the non-significant difference on various days of treatment. Collectively, all groups showed mean ± standard error of EPG on all days as 401.867±46.93. The lower bound of EPG on all days was 293.632 and the upper bound was 510.101.

The mean ± standard error of EPG of groups 1, 2 and 3 of various days with the upper and lower bounds are given in the above table. The values show that CAE of neem shows a mean and standard error of 764.000±81.295 on various days before and after treatment which portrays that the highest egg count remained in group 1 treated with neem or the group of animals treated with neem proved less efficacy or less reduction in the faecal egg count.

Based upon the observed "means", the "means" for homogeneous groups or subsets are displayed. The error term is Mean Square (Error) = 33044.783. The values in the similar column (subset) depict that there is the similarity of effectiveness between those groups e.g. LEV, OXY and Co and other drug levamisole are in the same column which means both of the drugs have an almost similar effect on EPG. In this case significance value is 0.965 ($p > 0.05$), which means the non-significant difference of effect, exists between both drugs or simply almost similar effect is depicted by both of the drugs. While CAE of neem is in subset 2 and significance difference value is 1.000 which shows that $p>0.05$ or there is not any significant difference within subset 2. However, a significant difference exists between the drugs under subset 1 and under subset 2 e.g., LEV, OXY and Co and levamisole both have significant differences from CAE of neem.

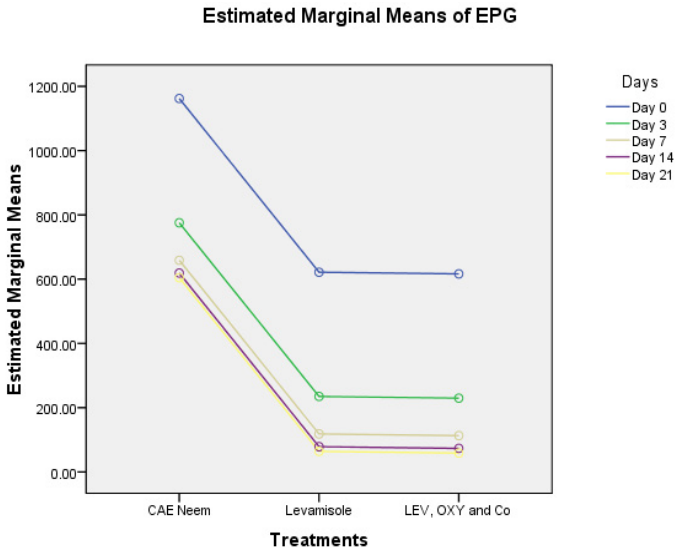

Figure 2: Graph showing decrease in faecal egg count of treated group.

The error term is Mean Square (Error) = 33044.783. As mentioned in previous table, the values in similar column (subset) depict that there is similarity of efficacy

on various days e.g., days 21, 14, 7 and 3 shown almost similar effect in the reduction of faecal egg count per gram. In this case significance value is 0.309 ($p > 0.05$), which means insignificant difference of effect, exists on these days or simply almost similar reduction in EPG is depicted on days 21, 14, 7 and 3. While on day 0 (pre-treatment) significance difference value is 1.000 which shows that $p > 0.05$ or there is not any significant difference within the subset 2. However, significant difference exists between the days under subset1 and under subset 2 e.g., days 3, 7, 14 and 21 (all these days of subset 1) have significant difference in reduction of EPG as compared to day zero (Figure 2, 3, 4, 5, 6 and 7).

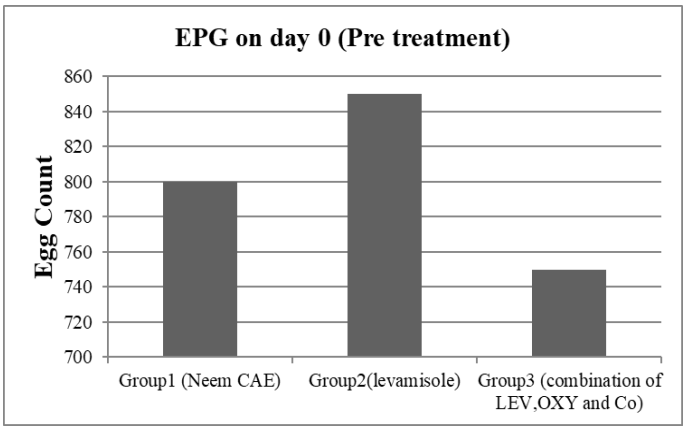

Figure 3: Graph showing faecal egg count of infected groups at day 0 pre treatment.

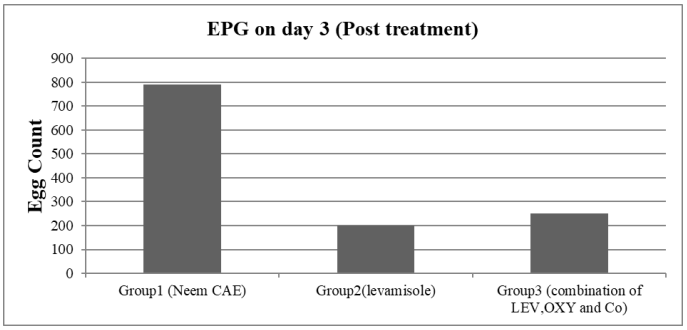

Figure 4: Graph showing decrease in faecal egg count of treated groups at day 3.

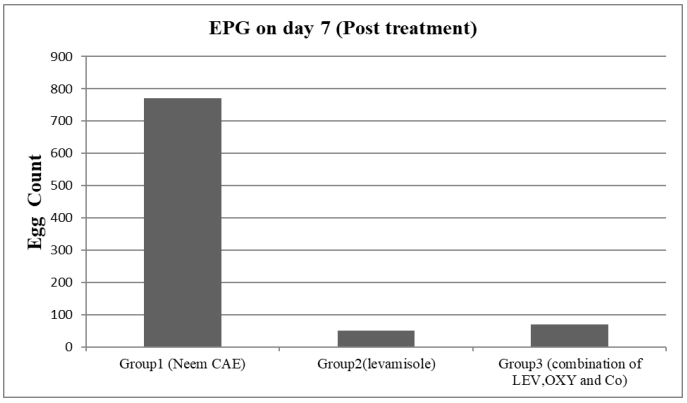

Figure 5: Graph showing decrease in faecal egg count of treated groups at day 7.

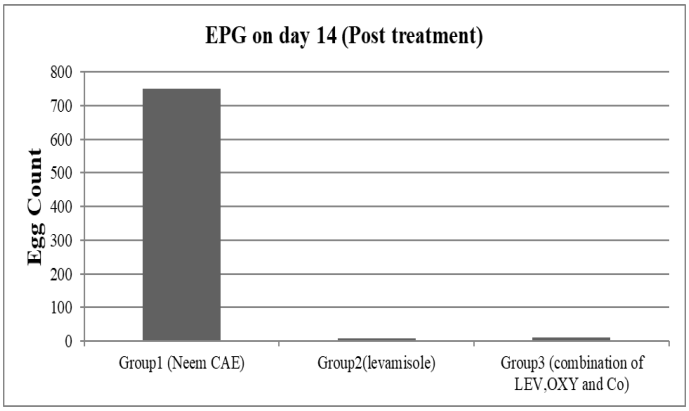

Figure 6: Graph showing decrease in faecal egg count of treated groups at day 14.

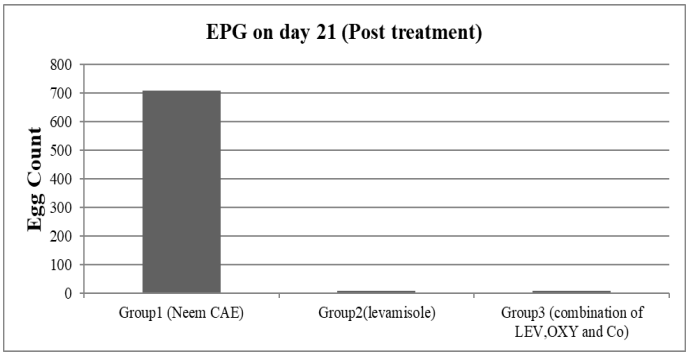

Figure 7: Graph showing decrease in faecal egg count of treated groups at day 21.

Faecal culture

The proportion of sheath tail extension is one of the most important criteria for the identifying the of larvae from faecal culture. The larvae culture of each infected cattle for identification was carried out on day 0 (pre-treatment) and days 3, 7, 14 and 21 (post treatment). There was a gradual decrease in larvae formation.

Mean of faecal larvae of nematodes of GIT on various groups on various days

The mean larvae count of various prevalent species on days 0 of groups 1, 2 and 3 are given in the form of (Tables 3, 4, 5, 6, 7 and 8).

Table 3: Mean larval count of GINs on day 0 of various groups.

Larval species	Medicated groups		
	1	2	3
Ostertegia ostertagii	340	360	310
Heamonchus spp.	320	330	305
Cooperia oncophora	70	85	60
Cooperia punctata	80	90	70
Trichuris globulosa	30	40	35
Oesophagostomum radiatum	35	45	40
Strongyloides papillosus	30	35	30
Trichostrongylus spp.	25	40	35

Table 4: Mean larval count of GINs on day 3 of various groups.

Larval species	Medicated groups		
	1	2	3
Ostertegia ostertagii	330	160	140
Heamonchus spp.	310	130	130
Cooperia oncophora	65	35	25
Cooperia punctata	75	40	30
Trichuris globulosa	25	15	15
Oesophagostomum radiatum	30	20	25
Strongyloides papillosus	20	15	15
Trichostrongylus spp.	25	15	20

Table 5: Mean larval count of GINs on day 7 of various groups.

Larval species	Medicated groups		
	1	2	3
Ostertegia ostertagii	325	60	50
Heamonchus spp.	300	40	40
Cooperia oncophora	60	15	15
Cooperia punctata	70	20	20
Trichuris globulosa	20	10	15
Oesophagostomum radiatum	25	15	10
Strongyloides papillosus	20	10	15
Trichostrongylus spp.	25	10	10

Table 6: Mean larval count of GINs on day 14 of various groups.

Larval species	Medicated groups		
	1	2	3
Ostertegia ostertagii	320	20	25
Heamonchus spp.	295	10	15
Cooperia oncophora	65	10	05
Cooperia punctata	70	15	10
Trichuris globulosa	30	05	05
Oesophagostomum radiatum	25	10	05
Strongyloides papillosus	20	05	10
Trichostrongylus spp.	25	05	05

Table 7: Mean larval count of GINs on day 21 of various groups.

Larval species	Medicated groups		
	1	2	3
Ostertegia ostertagii	300	07	10
Heamonchus spp.	290	03	06
Cooperia oncophora	65	01	03
Cooperia punctata	75	02	04
Trichuris globulosa	25	01	01
Oesophagostomum radiatum	30	01	02
Strongyloides papillosus	25	01	01
Trichostrongylus spp.	20	01	02

Table 8: Percentage diminution in larval count on day 21 as compared to day zero (0).

Larval species	Percentage decrease in larval count of medicated groups		
	1	2	3
Ostertegia ostertagii	11.765%	98.050%	96.774%
Heamonchus spp.	09.375%	99.090%	98.033%
Cooperia oncophora	07.143%	98.820%	95.00%
Cooperia punctata	06.250%	97.777%	94.28%
Trichuris globulosa	16.666%	97.500%	97.143%
Oesophagostomum radiatum	14.286%	97.777%	95.00%
Strongyloides papillosus	16.666%	97.143%	96.666%
Trichostrongylus spp.	20.00%	97.500%	94.285%

The pattern of the reduction in mean larval count was depicted slightly different but almost similar to the pattern shown by drugs on faecal egg count reduction. The maximum reduction was on day 21 by the group 2 drug levamisole, followed by the drug LEV, OXY and Co of group 3 and the CAE of 'neem' was least effective by showing the least reduction in mean larval count (Figures 8, 9 and 10). The break up is given as per the following (Table 9).

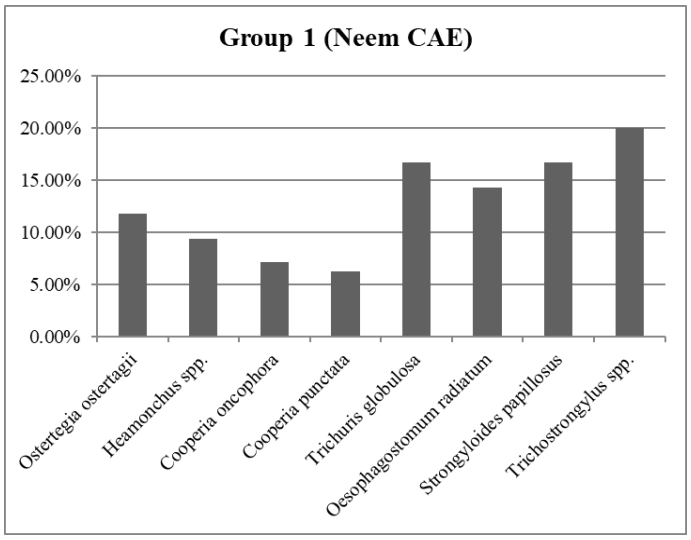

Figure 8: Graph showing percentage larval decrease of various species by group 1.

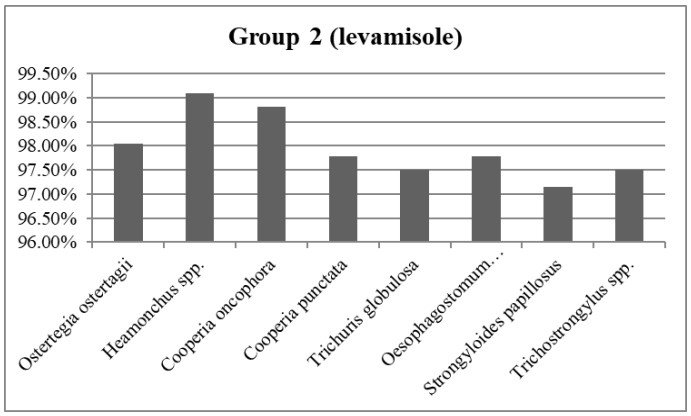

Figure 9: Graph showing percentage larval decrease of various species by group 2.

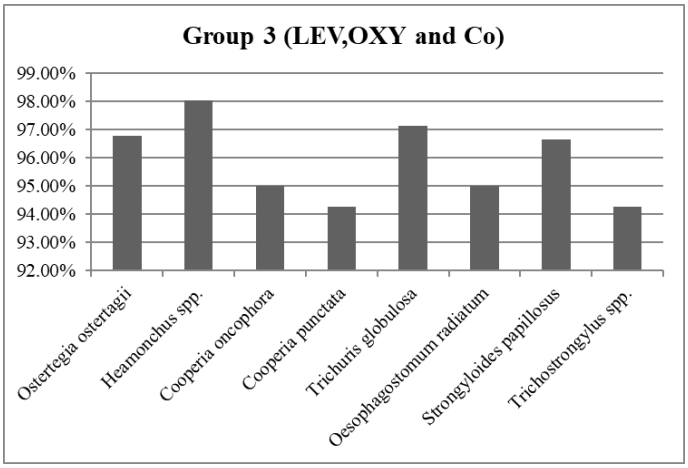

Figure 10: Graph showing percentage larval decrease of various species by group 3.

Table 9: Average percentage decrease in larval count on day 21 as compared to day zero.

Groups	Average percentage (%) reduction in mean larval count
1 (CAE of neem)	12.8%
2 (levamisole)	97.97%
3 (LEV+OXY+Co)	95.9%

The maximum effect of the group 1 drug divulged on day 21 was against *Trichostrongylus* spp. which was 20%. The highest effect of the group 2 drug was revealed on day 21 against *Heamonchus* spp. which was 99.09%. The highest effect of the group 3 drug was exposed on day 21 against *Heamonchus* spp. which was 98.033%. As a whole, the highest efficacy or highest reduction in mean larval count was delineated by group 2 on day 21. The graph given below explains the average percentage decrease of various larvae on day 21 as compared to day zero (Figure 11).

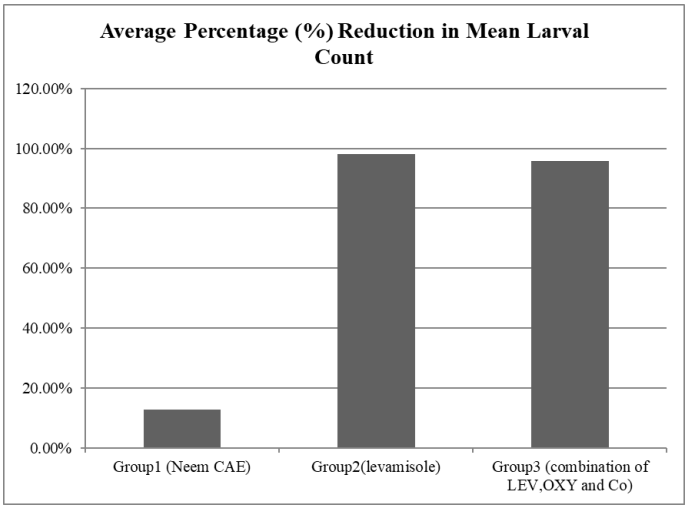

Figure 11: Graph showing average percentage decrease in larval count on day 21 as compared to day zero (0).

It has been observed that "Neem" has reduced the propensity by decreasing the cell growth (Bhola *et al.*, 2014). If "Neem" is applied with due care, it can be useful as pesticide and as an insecticide without side effects (Boeke *et al.*, 2004). "Neem" seed oil has been figured out as a pesticide and parasitical (Chaudhari *et al.*, 2013). The results of the contemporary studies have revealed and suggested that the crude extracts of neem could be used as a natural medicine (Hossain *et al.*, 2013).

Neem can be used effectively to mitigate or alleviate the growth and survival of the parasites (Senthil *et al.*, 2007). The tree of "Neem" (*Azadirachta indica*) contains secondary metabolites that demonstrate a wide range of biological activities against the parasites. The studies may not have been made on such magical plants, nevertheless, the neem plant has tremendous beneficial health effects. For the reason that neem decreases parasitic load considerably upon the domestic animals to be protected for a large part. The "neem" based anthelmintics have a tremendous potential and prospective interest for dairy breeders (Habluetzel *et al.*, 2007). After their studies, Boeke and colleagues described that the "neem" (*Azadirachta indica*) tree provides numerous beneficial extracts that are used for health vintages in terms of blood sugar lowering properties, anti-parasitic, anti-inflammatory, anti-ulcer and hepato-protective effects. It has been assessed about the toxicological data from animal and human experiments with oral administration of different 'Neem' (*Azadirachta indica*) based preparations. In a nut shell, the "neem" has tremendous potential being an insecticidal and parasiticidal. Moreover, the nutritional components like selenium and cobalt may be present (Boeke *et al.*, 2004).

Conclusions and Recommendations

In a nut shell, in folk use mostly water decoction of neem seeds is used, it is effective but the efficacy is so slight in comparison with synthetic anthelmintics that it is negligible. In this scenario, the CAE of neem seeds may be more useful for the farmers holding small herds or may be facing diffidence limitations. On the anthelmintic efficacy, the seeds of the neem (*A. indica*) appear to have a great potential for upcoming large-scale research in the realm of one health including public health.

Acknowledgements

The Staff of Department of Veterinary Medicine, UVAS, Lahore, Pakistan has provided technical support for research especially carrying out the experiments.

Conflict of interest

The authors have declared no conflict of interest.

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