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EFFICACY OF NEEM (*AZADIRACHTA INDICA*), SHAHTRA (*FUMARIA PARVIFLORA)* LEAVES AND KALONJI *(NIGELLA SATIVA*) SEEDS AGAINST HAEMONCHUS *CONTORTUS* INFECTION IN LOCALLY BRED RAMBOUILLET SHEEP IN PAKISTAN

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ABSTRACT

Sheep farming is the backbone of Pakistan's rural economy and *haemonchosis* is a major impediment in its way. The objective was to evaluate the efficacy of three medicinal plants *Azadirachta indica* (Neem), *Fumaria parviflora* (Shahtra) leaves and *Nigella sativa* (Kalonji) seeds against *Haemonchus contortus* (*H. contortus*) infection in locally bred *Rambouillet* sheep. One hundred and ten naturally infected female sheep diagnosed on the basis of identification of the parasite and faecal egg count, were selected. The animals were assigned to 11 treatments in a completely randomized (CR) design with 10 animals per treatment. The treatments were Albendazole 7.5 mg/kg BW (positive control), Neem leaves extract (NLE) 50 mg/kg BW, NLE 100 mg/kg BW, NLE 150 mg/kg BW, Shahtra leaves extract (SLE) 50 mg/kg BW, SLE 100 mg/kg BW, SLE 150 mg/kg BW, Kalonji seeds extract (KSE) 50 mg/kg BW, KSE 100 mg/kg BW, KSE 150 mg/kg BW and un-treated infected (Negative control). All the extracts were prepared in aqueous solution. Treatments were administered as single dose orally. Faecal egg count was recorded on zero, 7th, 14th, 21st and 28th-day post-treatment for analysis. Faecal egg count reduction percentage (FECR%) was recorded to assess the efficacy of the drugs. None of the treatments could fully eliminate faecal egg production by the 28th-day post-treatment. Albendazole showed significantly higher FECR% as compared to Shahtra and Kalonji treated groups ($p \leq 0.01$). FECR% on day 28 showed no significant difference between Albendazole, NLE 100 and NLE 150 ($p > 0.05$). The three medicinal plant extracts showed a limited efficacy against *H. contortus* indicated by FECR% when compared with untreated animals. However, the efficacy of NLE 100 was closest to Albendazole showing its potential as an anthelmintic ($p > 0.05$).

Keywords: Haemonchus; Sheep; Neem; Shahtra; Kalonji.

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INTRODUCTION

The livestock sector is a vital segment of the economy throughout the world particularly in developing countries and the sheep industry is its major contributor. Over 1.2 billion sheep population in the world and its products annually generates a trading volume of 9.44 billion US dollars (FAO 2018). Pakistan has a sheep population of 31.6 million (M) and produced 4.56 M tons sheep meat in 2019 having an export value of 30.25 M US dollars (FAO 2019). The demand for sheep products like organic meat, milk, cheese, wool and skins is increasing because of the rapidly multiplying human population. However, the industry remains exposed to various ecto- and endo-parasites, among which *Haemonchus contortus* (*H. contortus*) or Barber pole infection is still a major challenge. The problem is even more grave in tropical and subtropical regions where this parasite finds ideal climatic conditions for its propagation during hot and humid parts of the year. The nematodirus parasite feeds on the blood of sheep causing anaemia, anorexia, oedema, weight loss and even deaths of lambs thus adversely affecting production and farmer's profitability. Several conventional anthelmintic drugs are used for its prevention and cure. The organism is developing resistance against almost all available classes of anthelmintics due to extensive pressure leading to genetic modification. Other than increasing the cost of producing these synthetic drugs have an undesirable hazard of having their residual effects in consumable and usable products which necessitates research to find alternate compounds (Perry and Randolph 1999).

Crude powders, derived compounds and extracts of medicinal plants have traditionally been used against parasitic diseases in most parts of the world. Such natural compounds are also finding their therapeutic place in organic farming. A number of studies have been carried out on their use with confronting findings; however, most of these studies were carried out *in vitro*. *Azadirachta indica* (Neem) is a popular fast growing shady tree abundantly grown in tropical and sub-tropical countries. Compounds extracted from its leaves, seeds and bark are known to have medicinal value and are used as topical applications as well as against -ecto and endoparasites

both in animals and humans (Akbar 2020, Ezzat *et al*. 2018). *Fumaria parviflora* (*F. parviflora*), called 'Shahtra' in Indo-Pakistan, is an annual herb found worldwide and used for various gastrointestinal ailments and as anthelmintic in the Unani system. *Nigella sativa* (N. sativa) called Kalonji in Indo-Pakistan is another famous herb known for long in folk history for its wide range of therapeutic effects due to its bioactive compound 'thymoquinone' (Ahmad *et al*. 2013). The objective of the current experiment was to check the comparative efficacy of aqueous extracts of these three herbs and a conventional anthelmintic against *H. contortus* on locally bred Rombouillet sheep. Number of research articles have been published on anthelmintict effect of these plants most of which have been carried out *in vitro* (Azra *et al*.2019, Selvaraju and Dhanraj 2019, Sindhu *et al*. 2014,). This is the first-ever comparative study carried out on an exotic sheep breed in Pakistan.

MATERIALS AND METHODS

Herbal Extracts: Fresh Neem leaves were plucked from the Neem trees standing in large numbers and easily available at Mona Depot, Tahsil Malakwal, District Mandi Bhauddin, Pakistan. The leaves were thoroughly washed with distilled water, chaffed and dried under a naturally ventilated shed at environmental temperature 37-41°C. The dried leaves were ground to powder in an electrical grinder. Aqueous extract of the crude powder was prepared as per the method described by Parida *et al*. (2002). Briefly, a 10 % aqueous solution of the crude powder was made with phosphate buffered saline and centrifuged at 3000 rpm for 10 minutes. The supernatant so obtained was passed through 0.22-micrometre filter paper and the extract so recovered was stored at -20°C. Before using, the Neem leaves extract (NLE) was suspended in distilled water to make a stock solution having a concentration of 100 mg/mL. The dosage volume of suspension for each treatment was calculated as per the bodyweight of individual animals (Table-1) and administered as an oral drench as a single dose. The dose rates of herbal extracts were selected basing on previous studies on similar herbs producing positive effects (Rana, 2015, Khan, 2015). Same dosage rates of aqueous extract of Phyllanthus Niruri were found hepato protective and effective anti oxidant in mice (Chatterjee and Sil, 2006). *F. parviflora* (Shahtra) leaves and *N. sativa* (Kalonji), were purchased from the local market of Lahore and aqueous extract was obtained in a similar manner. The plants were got identified from the Institute of Botany, University of the Punjab, Lahore.

Table 1. Treatment plan and anthelmintics.

Experimental Group	Extract/anthemintic and dosage mg/kg			
	***Azadirachta indica* (Neem) leaves aqueous extract (NLE)**	***Fumaria parviflora* (Shahtra) leaves aqueous extract (SLE)**	***Nigella sativa* (Kalonji) seeds aqueous extract (KSE)**	**Albendazole**
A (Positive control)	0	0	0	7.5
B1	50	0	0	0
B2	100	0	0	0
B3	150	0	0	0
C1	0	50	0	0
C2	0	100	0	0
C3	0	150	0	0
D1	0	0	50	0
D2	0	0	100	0
D3	0	0	150	0
E (Negative control)	0	0	0	0

Experimental design, animal husbandry and accommodation: The experiment was conducted on locally bred female Rambouillet sheep at Mona Depot Sheep Farm, District Mandi Bhauddin, Punjab, Pakistan from April to August 2018. All the animals were already having an individual tag number for identification. Routine vaccinations against Enterotoxemia, Anthrax and Black Quarter were carried out as per the farm policy, however; deworming schedule of all the flocks for the current year was withheld till the selection of experimental animals on the request of authors. Three hundred adult female sheep of 1-2 years of age having conjunctival anaemia score >3 (Kaplan *et al*. 2004, Ferreira *et al*. 2019), were preliminarily earmarked for the experiment. The sheep of each treatment group were housed in a pen with dimension 6x3x1.5 m temporarily erected with wooden planks under a naturally ventilated steel shed having a grooved concrete floor. Each pen was provided with a feeding trough and water tub for round the clock availability of green fodder and water. Overhead, electric fans were also installed for cooling. The pens were randomly allotted to treatments. Two

weeks adaptability period was given before starting treatment. The animals were not sent for grazing during the adaptability and experimental period. Concentrate ration composing of 0.250 kg gram, 0.250 wheat bran, 0.028 kg bone meal and 0.001 kg salt was offered to each animal daily at 07.00 h and 17.00 h. *Ad libitum* chaffed green fodder was provided. The animals were daily watched by a veterinary officer for the presence of any ailment and weighed two days before treatment for calculation of dose. Faecal samples were collected manually from rectums using sterile plastic gloves in the morning and marked for identification. Naturally infected cases out of this flock were diagnosed on the basis of faecal egg count through modified McMaster technique (Paras *et al*. 2018, Taylor *et al*. 2007, Whitlock 1948,) and *H. contortus* eggs were identified according to the procedure described by GnoRGi and McCuLLOCH (1989). Random faecal samples were collected pooled and subjected to larval culture as per the procedure described by Coles *et al*. (2006), Zarlenga *et al*. (2016) and Aguilar-Marcelino *et al*. 2020. As per this method, faecal samples were cultured and incubated in Petri dishes at 27°C for one week. The larvae were collected after adding Lugol's iodine solution and identified to be *H. contortus* according to MAFF (1986). The animals having eggs per gram (EPG) of more than 150 with more than 90% *H. contortus* ova were declared positive for haemonchosis. Out of this infected stock one hundred and ten animals were selected and weighed. The body weight (BW) ranged from 35-42 kg with an average of 41 ± 5.2 kg. The animals were assigned to 11 treatments in a completely randomized (CR) design with 10 animals per treatment. Detail of treatments is given in Table-1. The experimental protocol described by Wood *et al*. (1995) was followed. Faecal samples were collected from the individual animal in individual sterile sample bottles directly from the rectum before offering concentrate feed, sealed, marked and transported to Army College of veterinary sciences (ACVS) laboratory Sargodha in Iceboxes where these were stored under refrigeration till completion of further process. Faecal egg count was recorded on zero, 7th, 14th, 21st and 28th day after treatment for analysis and expressed as EPG. Faecal egg count reduction percentage (FECR) was recorded as following to assess the efficacy of the drugs (Levecke *et al.* 2018, Coles *et al*. 2006, Moskey and Harwood 1941).

$$\text{Efficacy\%} = \frac{\text{E b t}_1 - \text{E a t}_1}{\text{E b t}_1} \times 100$$

Statistical Analysis: Data were analyzed by software Statistical Package for Social Sciences SPSS version 20 (Spss 2011). EPG and FECR % values measured over time in each group that is 0, 7th, 14th, 21st and 28th day after treatment were analyzed through repeated measure analysis of variance in the general linear model. Differences among treatments were analyzed through one-way analysis of variance and means were separated by least significant difference and Tukey's test. Results were considered significant at $p \leq 0.05$ and to have a trend of significance at $p \leq 0.10$.

RESULTS

Table-2 and Table-3 show the means of faecal EPG and FERC% respectively on Day zero, 7, 14 and 21 whereas Table -4 and Table-5 show the same means on Day zero and 28. The faecal EPG of each group differed significantly ($p \leq 0.05$) over time, however; no significant ($p > 0.05$) difference was observed among the treatments on each recording day. Other than the negative control (no treatment; $p = 0.89$), FERC% also differed significantly ($p \leq 0.05$) in all the treatment groups over time. FERC% of various treatments on each day of observation was found to be significantly different ($p < 0.05$). However, comparison of FERC% means through least significant difference and Tukey's test showed no significant ($p > 0.05$) difference between (Albendazole), Neem leave extract 100 (NLE 100) and NLE 150 on day 14, 21 and 28. Three animals of Shatrah (SLE) 150 group developed ruminal tympany following treatment and were treated accordingly.

Table-2. Means of fecal EPG ± SEM of various treatments administered against *Haemonchus contortus* infection in sheep.

Experimental Group	Treatment mg/Kg	Days after treatment				Tx Trt P-value
		0	7	14	21	
A (Positive control)	Albendazole 7.5	2654.00±394.08	1978.60±296.21	1714.90±269.6	1355.80±204.17	0.00
B1	NLE 50	2527±553.12	2182±448	2123.4±441.58	2076.2±443.81	0.00
B2	NLE 100	2531.9±419.89	1905.6±353.13	1674.4±290.69	1414.4±244.63	0.00
B3	NLE 150	2547.1±507.93	1515.4±423.60	1470.9±417.34	1422.8±403.49	0.00
C1	SLE 50	2293.5±461.33	2016.1±410.81	1948.5±399.63	1878±385.90	0.00
C2	SLE 100	1830±307.29	1613±288.93	1508.2±266.67	1436.2±255.46	0.00
C3	SLE 150	2345.5±670.79	2042.2±624.55	1948.7±611.69	1858.1±577.92	0.00
D1	KSE 50	2589.4±389.17	2355.2±356.14	2271±344.17	2230.9±339.78	0.00

D2	KSE 100	2069.9±367.25	1907±347.02	1808.2±335.86	1742.2±315.03	0.00
D3	KSE 150	2113.2±442.14	1813.2±394.16	1731.6±382.70	1635.5±356.54	0.00
E (Negative control)	0	1845±408.45a	2840.3±397.25	2920.5±444.38	2832.5±404.90	0.00
P-value		0.95	0.631	0.378	0.17	

NLE = Neem leave extract, SLE= Shahtra leave extract, KSE=Kalonji seed extract
Tx Trt=Time x treatment interaction

Table-3. Means of FECR % ± SEM of various treatments administered against *Haemonchus contortus* infection in sheep.

Experimental Group	Treatment mg/Kg	Days after treatment				T x Trt P-value
		0	7	14	21	
A (Positive control)	Albendazole 7.5	0	24.72±2.49^{a}	35.72±1.86$_{a}$	49.08±1.31^{a}	0.00
B1	NLE 50	0	11.86±2.41^{b}	14.92±2.31^{b}	18.15±2.07^{bd}	0.00
B2	NLE 100	0	25.97±2.41^{a}	34.441±1.57^{a}	44.66±1.50^{a}	0.00
B3	NLE 150	0	39.06±1.89^{c}	41.54±1.68^{a}	43.81±1.^{65a}	0.00
C1	SLE 50	0	12.81±1.34^{b}	16.05±1.27^{b}	19.41±1.30^{bd}	0.00
C2	SLE 100	0	13.19±1.71^{b}	18.83±2.15^{b}	23.17±2.62^{bd}	0.00
C3	SLE 150	0	16.64±2.52^{b}	21.94±2.96^{b}	25.46±2.92^{b}	0.00
D1	KSE 50	0	9.10±1.17^{b}	12.48±1.02^{b}	14.17±0.85^{d}	0.00
D2	KSE 100	0	8.51±1.03^{b}	13.76±1.33^{b}	17.06±1.63^{d}	0.00
D3	KSE 150	0	15.53±1.70^{b}	19.71±1.87^{b}	23.95±1.50^{bd}	0.00
E (Negative control)	0	0	-1.26±2.55^{d}	-1.24±2.58^{b}	0.33±1.50^{e}	0.89
P-value		Not carried out	0.00	0.00	0.00	

NLE = Neem leave extract, SLE= Shahtra leave extract, KSE=Kalonji seed extract
Mean values in the same column with different superscripts differ significantly ($p<0.05$)
Tx Trt=Time x treatment interaction

Table-4. Means of fecal EPG ± SEM of various treatments on Day 0 and 28 against *H.contortus* infection in sheep.

Experimental Group	Treatment mg/Kg	EPG		Significance on Day 28 Versus	
		Day 0	Day 28	Negative control	Positive control
A (Positive control)	Albendazole 7.5	2654.00±394.08	1184.90±184.92	Sig*	--
B1	NLE 50	2527±553.12	2035.80±431.35	N.S	N.S
B2	NLE 100	2531.9±419.89	1298.54±217.31	N.S	N.S
B3	NLE 150	2547.1±707.93	1397.90±398.68	N.S	N.S
C1	SLE 50	2293.5±461.33	1299.97±303.40	N.S	N.S
C2	SLE 100	1830±307.29	1382.90±245.28	N.S	N.S
C3	SLE 150	2345.5±670.79	1743.10±546.15	N.S	N.S
D1	KSE 50	2589.4±389.17	2182.10±333.78	N.S	N.S
D2	KSE 100	2069.9±367.25	1691.60±306.40	N.S	N.S
D3	KSE 150	2113.2±442.14	1580.50±349.90	N.S	N.S
E (Negative control)	0	1845±408.45	2819.90±405.98	-	Sig*

N.S = Non significant (p>0.05), Sig*=Significant (p<0.05), NLE = Neem leave extract,
SLE= Shahtra leave extract, KSE=Kalonji seed extract

Table-5. Means of faecal FECR % ± SEM of various treatments on Day 28 against *H.contortus* infection in sheep.

Experimental Group	Treatment mg/Kg	FECR %		Significance on Day 28 Versus	
		Day 0	Day 28	Negative control	Positive control
A (Positive control)	Albendazole 7.5	0	55.92±1.69	Sig***	---
B1	NLE 50	0	19.52±2.33	Sig***	Sig***
B2	NLE 100	0	51.50±1.21	Sig***	N.S

B3	NLE 150	0	49.36±1.98	Sig***	N.S
C1	SLE 50	0	21.53±1.38	Sig***	Sig***
C2	SLE 100	0	26.16±2.58	Sig***	Sig***
C3	SLE 150	0	30.76±3.07	Sig***	Sig***
D1	KSE 50	0	16.23±1.02	Sig***	Sig***
D2	KSE 100	0	19.52±1.45	Sig***	Sig***
D3	KSE 150	0	26.90±1.66	Sig***	Sig***
E (Negative control)	0	0	0.94±1.90	--	Sig***

N.S = Non significant ($p>0.05$), Sig*=Significant ($p<0.05$), Sig**=Significant ($p<0.01$)
Sig***=Significant ($p<0.001$), NLE = Neem leave extract, SLE= Shahtra leave extract, KSE=Kalonji seed extract

DISCUSSION

In this experiment, none of the treatments could reduce the faecal EPG to zero by the 28th day of treatment. However, the highest reduction was noticed in positive control, the Albendazole group. Closer to Albandazole were the efficacy of NLE 100 and NLE 150. Although numerically FERP % was higher in Albendazole treated animals, however; the results did not significantly differ from those given NLE 100 and NLE 150. No significant difference was observed between NLE 100 and NLE 150. FECR % of all other treatments including the NLE 50, was significantly lower than Albendazole and NLE 100 or NEL 150. These findings about anthelmintic activity of NLE are in line with the number of earlier research works. Amin *et al*. (2010) reported a highly significant (p<0.05) reduction (37.60-47.03%) in EPG of gastrointestinal nematodes on the 7th, 14th and 21st day after treatment with NLE as compared to betel leaf (6.43-14.00%), devil's tree (3.04-11.04%), jute (0.50-5.26%) and turmeric (0.46-8.30), in sheep. However, no conventional anthelmintic (positive control) was used by Amin *et al*. (2010) for comparison. Previous studies have also found a significant ovicidal effect of Neem extracts against Teladorsagia (Ostertagia) circumcincta eggs (Al-Rofaai *et al*. 2012), inhibition of Gastrothylax indicus larva motility (Aggarwal *et al*. 2016) and reduced egg hatchability as well as adult motility of *H. contortus in vitro* when compared with Albendazole. Our results are also in close agreement with a recent study carried out by Sakti *et al*. (2018), who reported a similar (p>0.05) EPG (425 vs. 663)in naturally infected *H. contortus* positive ewes, treated with either 8% aqueous solution of *Azadirachta indica* (Neem) leaves or Albendazole, after 6 weeks of treatment. The findings of our experiment also substantiate results of Azra *et al*. 2019 who reported 87% mortality of *H. contortus* larvae, *in vitro,* by using NLE as compared to 67% noted by use of garlic bulbs extract. Neem (*Azadirachta indica*) is a native tree of Pakistan and India. Azadirachtin is the water-soluble active component present in a concentration of 0.022% in its leaves (Radhakrishnan *et al*. 2010). This substance kills the target parasite by disrupting its vital functions of metamorphosis, fertility and growth (de Souza Chagas and da Silva Vieira 2007). Moreover, the 'Tannin' content of the Neem leaf also has anthelmintic effect. Cuticle of the *H. contortus* gets disrupted by the condensed form of tannin in the leave extract which binds to the cuticle glycoproteins (Sakti *et al*. 2018, Naz *et al*. 2013). Consequent reduction in flavnoid diffusion, increased exposure to the compound and inhibition of enzyme secretions causes paralysis and death of the parasite (Tresia *et al*. 2016, Kerboeuf *et al*. 2008). The other bioactive compounds found in Neem plant which have anthelmintic properties include quercetin, polyphenolic flavonoids, alkaloids, ßsitosterol, glycosides and saponins (Alzohairy 2016). The highest average FECR % with the use of NLE extract against sheep *H. contortus* in this experiment (51.50%) remained lower than Hamad (2018) (85.11%) in *a Vivo* trial. This could be due to a different source of 'azadirachtin' extract used which is seed kernels. Neem seed kernel is also rich in azadirachtin and yields 2.34% crude aqueous-methanol extract. Moreover, the dose used in their case was 4 grams per kg body weight. Contrary to our results, Hamad (2018) also found no effect of Oxfandazole in reducing faecal egg count due to the development of disastrous resistance against anthelmintic drugs, in *H. contortus* prevalent at the sheep farm under study. FECR % of Albendazole treated animals in the current study remained 55.92±1.69 % which is though highest among all other groups but lower than that reported (97.2%) by Seyoum *et al*. (2017), however; they carried out the said study on all types of gastrointestinal nematodes of sheep whereas in the present experiment the target organism was *H. contortus*. Moreover, the aforementioned study was carried out in Ethiopia and resistance levels might differ in the parasites.

In another experiment feeding of Neem leaf meal to infected sheep at 5% of concentrate, ration resulted in 88 % reduction in faecal egg count of treated animals after 12 weeks which signifies the anthelmintic efficacy of Neem leaves (Adelusi *et al*. 2019). In a similar study conducted by Iqbal *et al*. (2012), 98.91% reduction in EPG was recorded after 14 days of drenching the infected lambs with crude aqueous methanol extract of Neem seeds at the dose of 2g/kg body weight. These research findings substantiate our results and suggest that compounds derived from Neem from

leaves or seeds, as aqueous or ethanolic extracts have an anthelmintic role both *in vitro* and *vivo* and can be exploited for control of *H. contortus* infection particularly while the said organism is developing resistance against conventional chemicals. However, there is a need to carry out further research to determine the type of extract and dosage level which should be effective enough to control infection but have the least anti-nutritional effect. Such indigenous compounds can also economize sheep production and also have the potential to replace synthetic drugs in popular organic farming practices.

FECR% of Kalonji seed extract (KSE) treated animals in the present trial remained significantly lower than Albandazole and NLE. No specific study is available for use of Kalonji seeds against *H. contortus* in sheep for comparison of results. However, a number of therapeutic, antimicrobial, anti-oxidant and anthelmintic effects of Kalonji seeds have been reported due to its bioactive compound thymoquinone (Adegbeye *et al*. 2018, Ahmad, *et al*. 2013, Tembhurne *et al*. 2014). Al-Nakeeb *et al*. (2015) reported a significant scolicidal action of Kalonji seeds *in vitro* against protoscolices collected from hydatid cysts of sheep liver. Such efficacy could not be established in the current experiment.

Efficacy of SLE (*Fumaria parviflora*) against *H. contortus* in our experiment remained lower than the study conducted by Hördegen *et al*. (2006) who reported a 0.31 survival rate of 3rd stage *H. contortus* larvae collected from sheep after treatment with aqueous ethanol of the whole plant at the dose rate of 80 µg/µl *in vitro*. The reason for these results could be direct treatment *in vitro* in the aforementioned study. The potential of the Neem plant as an anthelmintic indicates that further research is required to extract/purify the compounds contained in it and determine its effective and safe dose for use against *H. contortus* in sheep.

Conclusion: This experiment has shown a limited efficacy of Neem, Shahtra and Kalonji plant extracts in naturally infected sheep against *H. contortus* indicated by faecal egg production reduction percentage when compared with untreated animals. However, the efficacy of aqueous extract of Neem leaves given at the dose rate of 100 mg/kg body weight was closest to Albendazole given at the recommended dose rate which itself failed to fully eliminate the egg production. Numerically, FERP % revealed higher efficacy of Neem followed the Shahtra and Kalonji.

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Statement of animal rights: All applicable international, national and institutional guidelines for care and use of animals were followed. The experimental protocol was approved by Animal Care and Use Committee, University of the Punjab, Lahore, vide number 2359/ACAD.

Conflict of Interest: The authors declare that they have no conflict of interest.

Authors' contribution: All authors contributed to the study conception and design as well as manuscript writing. Material preparation, data collection and analysis were performed by Muhammad Naeem. All authors read and approved the final manuscript for submission.

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