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MITICIDAL ACTIVITY OF METHANOLIC EXTRACT OF *VITEX NEGUNDO-LAM* AGAINST *SARCOPTES SCABIEI* IN ANIMALS AND MAN

M. A. Khan, A. H. Shah, A. Maqbol[*], N. Khan[**] and Z. U. Rahman

Department of Biological Sciences Gomal University D. I. Khan, KPK, Pakistan; [*]Department of Parasitology University of Veterinary and Animal Sciences Lahore, Pakistan; [**]Department of Weed Science KPK Agricultural University Peshawar, Pakistan.
Corresponding Author: email; drmuradwazir@yahoo.com

ABSTRACT

The present study was designed to determine the miticidal effect of methanolic extract from *Vitex negundo* Lam. at the rate of 10 and 20% concentrations through topical application on scabies affected skin of camel, buffalo, goat, dog and man. The ivermectin was used as reference compound and100% methyl alcohol as control group. The topical application of the treatment groups on scabies affected buffalos gave 43, 73 ,81 and 0%, on camels 46, 75, 84 and 0% on dogs 42, 77, 83 and 0%, on goats 42, 69, 81 and 0% and on man 61, 78, 84 and 0% protection, respectively, after 5th weeks of application. In addition, the effect of *Vitex negundo* methanolic extract with three different concentrations (10, 20, and 30%) was studied *in vitro* to determine the potential to kill *Sarcoptes scabiei*. The results revealed that 10, 20 and 30% concentrations of *Vitex negundo* methanolic extract caused 70, 80, and 90% mortality of the *Sarcoptes scabiei* mites, respectively in comparison to ivermectin and methyl alcohol that gave 85 and 5% mortality, respectively. It is concluded that methanolic extract of *Vitex negundo* Lam was found good as ivermectin a proven miticide both in *vitro* and *in vivo*.

Keywords: *Vitex negundo*, methanolic extract, mite, ivermectin, *Sarcoptes scabiei*.

INTRODUCTION

Scabies is one of the skin diseases in animals and humans. This disease is caused by a mite known as common itch mite (*Sarcoptes scabiei* L.). Scabies is a widely spread and a highly contagious disease throughout the world (Burgees, 1994). There are several types of this disease such as classical scabies, Norwegian scabies, and clean scabies. However, classical scabies is very common in people and animals in Pakistan and many other countries. It has been reported that approximately several millions of people and animals are affected by many skin diseases including scabies (WHO, 2001; Hicks MI and DM Elston, 2009; Lusat *et al*., 2009).

The gravid female of the parasite lays two to three eggs a day in burrows (several millimeters to several centimeters in length) created at the stratum corneum of epidermis. After 50-72 hours, the larvae emerge out of eggs and make new burrows. The larvae molt to nymph form and after two further molts, reach to adult life stage. This life cycle of *Sarcoptes scabiei* takes about 10-17 days to complete (Soulsby, 1982).

Bathing by water can remove a significant number of *Sarcoptes scabiei* mites from the affected people and animal's body; however, inappropriate bathing may affect the skins of the effected people and animals. Washing the bodies of people and animals infested with *Sarcoptes scabiei* mites may also cause skin dryness and scratches and may harm them because dry skin is more proven to severe itching and irritation. In such conditions people and animals affected with *Sarcoptes scabiei* may lead to other health problems such as dermatitis, pyoderma and sometimes eczema and urticaria. Treatment of *Sarcoptes scabiei* generally entails the application of topical creams for classical scabies; however, oral application of ivermectin is good to treat crusted scabies (Mounsey *et al*., 2008). Another medicine such as permethrin which is an active component of topical creams can also be used to treat scabies disease. In countries, such as Australia, permethrin has been successfully used for the treatment of scabies disease (Carapetis *et al.,* 1997). However, a recent *in vitro* study on acaricidal sensitivity has demonstrated increased tolerance of *Sarcoptes scabiei* mites to permethrin collected from indigenous people communities across northern Australia (Mounsey *et al*., 2009). Several constraints such as resistance of *Sarcoptes scabiei* to synthetic medicines that might come due to the repeated use of the same mode of action medicines in people and animals sick with scabies, side effects of medicines, their unaffordable cost for some people and the long residual properties of most of the synthetic medicines available in the market justify the cause to find alternative approaches to control *Sarcoptes scabiei* mite. One such approach could be the use of plants extracts that might be useful to cope with scabies disease on human and animals.

Five-leaved chaste tree (*Vitex negundo* Lam.) is large aromatic shrubby plant of Verbinaceae family with a slender plant stature grow in rainfed areas of southern

districts of Khyber Pakhtunkhwa province of Pakistan (Sahare *et al*., 2008a). This plant contains various chemical compounds such as alkaloids, tannins and flavonoids (Sahare *et al.,* 2008b). Traditionally the plant has been reported to have repellent as well as pesticidal properties, however, the plant has also been suspected for its medicinal values which may be useful for the treatment of various diseases (Liu *et al*., 2010).

The bio-medicinal properties of *Vitex negundo* against pathogens have been reported recently in several studies, such as Trapti *et al.* (2009) and Sahare *et al*. (2008b). In an earlier study, methanolic extracts of four species of *Vitex* (i.e. *Vitex negundo, Vitex trifolia, Vitex peduncularis* and *Vitex altissima*) have shown larvicidal efficacy against *Culex quinquefasciatus* larvae (Kannathasan *et al*., 2007). Other chemical compounds such as β-caryophyllene and α-pinine isolated from *Vitex negundo* have also shown insecticidal and repellent properties against greenfly (*Aphis gossypii* Glov. Homoptera: Aphididae) (Liu *et al*., 2010). Ethanolic extracts of *Vitex negundo* was also noticed to be effective against Indian earth worm (*Pheretima posthuma* Vaillant) (Trapti *et al.,* 2009). Keeping in view the above bio-medicinal properties of *Vitex negundo* against different diseases the present study was designed to evaluate the effects of methanolic extracts of *Vitex negundo* for the treatment of scabies affected people and animals.

MATERIALS AND METHODS

Processing of plant materials: The present study was designed to determine the miticidal effect of methanolic extract of *Vitex negundo* Lam against scabies affected animals and man. For that purpose plant of *Vitex negundo* was collected from district Bannu and karak of Khyber Pakhtunkhwa province. The plants were cut at soil level and the stems and leaves were collected and placed in brown paper bags during summer season. All the samples were transported immmediatly to the Laboratory, of Organic Chemistry Gomal University Dera Ismail Khan for onward processing. For extraction the standard experimental protocol of Daniel (1991) was adapted. The dried samples were grinded to powder form by using a grinding machine. The 500 grams powder was mixed with 2 liters of 100% methyl alcohol (CH_3OH) in a beaker. This mixture was stirred twice a day regularly for 20 days to mix the powder with CH_3OH to obtain the standard extraction of *Vitex negundo.* The methanolic extract was filtered through muslin cloth and collected in a beaker and stored in a refrigerator under 4 ^{0}C. The unfiltered powder residues were re-mixed with a similar quantity of CH_3OH as mentioned above and this practice was carried out two times to get complete extraction. The filtered mixture was stored in refrigerator. The extract was subjected to Rotavapour at 55-63 ^{0}C for 9-10 hours and achieved 5.2% crude extract out of the total extracted volume of *Vitex negundo*. The crude extract was stored in a refrigerator at 4 ± 1 ^{0}C for further use.

Preparation of the stock solutions: The methanolic extract of *Vitex negundo* (50 g) was weighed by using electronic balance and poured into 100 mL flask. The 50 ml CH_3OH was added to get the final volume 100 mL of methanolic concentration of *Vitex negundo*. This constituted 50% CH_3OH diluted extracts and designated as 50% stock solution. Three different concentrations of 10, 20 and 30%were prepared by diluting the standard stock solution serially with CH_3OH. The ivermectin at 0.2 mg/ kg^{-1} body weight of the individuals was used as a reference compound recommended for scabies treatment (Sparsa *et al*., 2006) while CH_3OH was used as control treatment group in the experiment.

Rearing of *Sarcoptes scabiei* mites: A mass culture of *Sarcoptes scabiei* was established on laboratory rabbits in the Faculty of Pharmacy Gomal University Dera Ismail Khan. The duration of experiment was extended from August 2008 to September 2009. The rabbit zone was provided with the prerequisites conducive environment for the rearing of mite (adequate oxygen supply, 21-25 ± 3^{0}C temperature and 65-71 ± 5% humidity. The rabbits were offered two times feed drinking water. No miticidal drugs were provided to the rabbits during this period of time.

Mites rearing and collection: Ten rabbits infested with *Sarcoptes scabiei* were kept for 30 days to get maximum typical skin lesions. After getting maximum lesion on the rabbits, the lesions were then scrapped using a scalpel and placed in Petri dishes. The scrapes were placed on glass slide and were mixed with 10%Potassium Hydroxide (KOH) of solution. The slide was placed under microscope for detection of mites. The following mixture was prepared to demonstrate the parasitic burden on individual rabbit. Three times greater quantity of sodium chloride (NaCl) solution was added to the actual quantity of skin lesions mass (10 grams) and mixed them together. The mixture was centrifuged at 1500 Rotations per minute. To identify the infestation population of *Sarcoptes scabiei* mites the supernatant of the centrifuged solution was discarded and a drop of the sediments was put on counting chamber slide to confirm the number of mites. The counting chamber slide was checked under Stereo Binocular Microscope (Model Sz2-ILST Sz61, Olympus, Tokyo, Japan). The sediment of the skin lesions having 50 mites per drop was used as standard infestation which is good for *Sarcoptes scabiei* live mites collection.

To collect *Sarcoptes scabiei* mites five infested female rabbits were confined separately in 5 linen bags (2x2 feet) covering its all parts except mouth, eye and nose. The vaginal and anal openings were connected to the plastic tube outside the linen bag. The mites were

collected every 24th hour from the linen bag and were transferred to rectangular cells (3x4 inches) containing nutrients (i.e. fish flakes, multivitamins, amino acids, glucose, skin shaving and horse serum semisolid globules). The house dust mite (*Dermatophagoides farinae* Hug.) collected from the common under use home carpets and beds were also added to these rectangular cells in order to provide suitable environment to the *Sarcoptes scabiei* mites for its rearing. The rearing media cells containing mites were tightly closed with bulldog clips. These cells were placed in a glass container on a steel gauze (covering the filled water in the 2 liters glass container) in order to keep cells away from direct water contact. The glass container was closed with a lid having only a small opening for Oxygen (O_2) supply. The container was kept in a room with a tightly closed door (18-24 ± 3 ^oC temperatures) with 65-71 ± 5% humidity for periodic use.

Structure of cell for enclosing mite and food: All rectangular cells used to house the mites were constructed from concave black plastic discs with a varnish attached black filter paper at the opening of the discs to provide sufficient relative humidity to the residing mites. The mites along with food were enclosed in the hollow section of the discs covered by glass square and tightly closed using Vaseline and bulldog clips.

To feed the experimental mites, aquarium gold fish flakes and tetra-mine tropical fish flakes were provided once a week and were shaken on weekly basis to activate the mites. Optimum climatic conditions such as 25 ± 3 ^oC temperature and 65-70 ± 5% humidity (Hallas, 1991; Arlian and dippold, 1996) for the growth of mites (*Sarcoptes scabiei*) were maintained inside the Postgraduate Microbiology research Laboratory in the department of Biological sciences Gomal University D.I.Khan. All Cells were placed in sealed desiccators and humidity was provided by mixing KOH and water placed at bottom of desiccators (70 pellets of KOH + 85 mL tap water to provide 65% RH at 25 ^oC) inspected on weekly basis. Further the desiccators were placed in growth cabinet (10x8 feet) having 65% RH and 25 ^oC temperature for more assurance of providing suitable environment for *Sarcoptes scabiei* mites.

Media preparation for *Sarcoptes scabiei:* To provide required nutrients to the *Sarcoptes scabiei* mite during experimental trial a media was prepared adapting methodology of Brimer *et al.,* (1993). This media was warmed using a spirit lamp and normal horse serum was added into it. The media was once more warmed and autoclaved at 121 ± 4 ^oC for 10 minutes to eradicate the undesired micro organisms, then added human skin shavings (mites feeding agent) and yeast (*Sachhromyces cervisae*) to discourage the growth of other microorganism and was incubated at 37 ^oC for 24-48 hours to make it stable for use *in vitro* technique.

Anti-scabies screening through *in Vitro* technique: The experiments were performed using 96 wells prepared tissue culture plates made of polyethylene. To each well of tissue culture plate 3.5 mg of prepared media *for sarcoptes scabiei* was added. An adequate dilution (30µl) of 10, 20 and 30% *Vitex negundo* extract was added into the bottom of the wells and allowed to dry for 24 hours at room temperature(37 ^oC) prior to launching the experiment . In the opposite wells 30µl of ivermectin (10 mg mL^{-1}), a proven acaricide was put and used as reference compound. The control wells were only added 30µl CH_3OH.

Into each well, 20 mites were released with the help of teasing needle while observed under stereo microscope. To avoid escape of mites, each well was covered with 16 mm diameter paper disk (porous) and was incubated at 21 ± 2 ^0C and 70 ± 4% RH for 48 hours with sufficient supply of oxygen. The incubator was manipulated inside dark room having relatively 21^0C ±2 temperature and 70% humidity. The mortality of mites was recorded with a Stereo Binocular Microscope after 24 hours incubation period. The criteria for conclusion of result was on the bases of movable (live) and non-movable (dead) observed under the Stereo Binocular Microscope and checked through teasing needle at three different time intervals (24, 48and 72 hours).

Anti-scabies screening using *in vivo* technique: For topical use on scabies affected animals, 10 and 20% concentration of methanolic extract of *Vitex negundo* in saturated CH_3OH was prepared by diluting 50% stock solution. The two concentrations (10 and 20%) of *Vitex negundo* extract as well as ivermectin (reference compound) and the pure methyl alcohol (control) were applied separately on 10 each scabies positive buffalos, camels, dogs, goats and persons at one and two weeks interval (i.e. on 1st, 7th, 14th and 28th day of experiment). The data was recorded on 7th, 14th, 28th and 35th day of each species.

Statistical design and analysis: Both the experiments were established using completely randomized design with three replications of all the treatments (10, 20 and 30% *Vitex negundo* methanolic extracts) for the *in vitro* experiment and both the treatments (10 and 20% *Vitex negundo* methanolic extracts) for the *in vivo* experiment. A reference compound treatment and that of a control was kept for comparison with the other treatments. A t-test was performed on the means of the data (at $P < 0.05$) to see if there were significant differences among the treatments.

RESULTS

The effect of *Vitex negundo* methanolic extract with three different concentrations (i.e. 10, 20, and 30%) revealed that the 10, 20 and 30% concentrations gave 70,

80, and 90 % mortality of *Sarcoptes scabiei* mites, respectively while ivermectin and control treatments showed 85 and 5% mortality of the *Sarcoptes scabiei* mites after 48 hours of application (Figure I). The different concentrations of *Vitex negundo* extract when compared with each other showed significantly different effect on the mortality of *Sarcoptes scabiei* mite ($P < 0.05$) (Table I). However, a significantly low mortality of *Sarcoptes scabiei* was recorded in the control treatments as compared to 10, 20 and 30% extracts of *Vitex negundo* solution and that of the ivermectin (Fig. I).

Efficacy of 10 and 20% methanolic extract of *Vitex negundo* topical use over the skin lesions of persons and animals was evaluated in comparison to ivermectin and that to the control. The miticidal activity of two concentrations (10 and 20%) methanolic extracts of *Vitex negundo* and ivermectin on scabies lesions in camels, buffalos, goats, dogs and peoples is compared with the control treatment (Figure II). The mean averages data recorded on the efficacies of 10 and 20% concentration of *Vitex negundo* was greater in case of 20% than the 10% concentration against the scabies lesions on the skin of, camels, buffalos, dogs, goats and peoples (Fig II). However, a significantly slow disappearance of *scabies lesions were* recorded in the control treatments as compared to 10, 20 % extracts of *Vitex negundo* solution and that of the ivermectin (Table II).The results indicate a significant difference ($P < 0.05$) between the two concentrations (10 and 20%) as well between concentration of *vitex negundo* (10 and 20%), ivermectin and control on 5th week (Table II). In addition, the statistically analysed results obtained of peoples versus camels, buffalos and dogs were non significant ($P > 0.05$).where as between peoples and goats was significant ($P < 0.05$) (Table III).

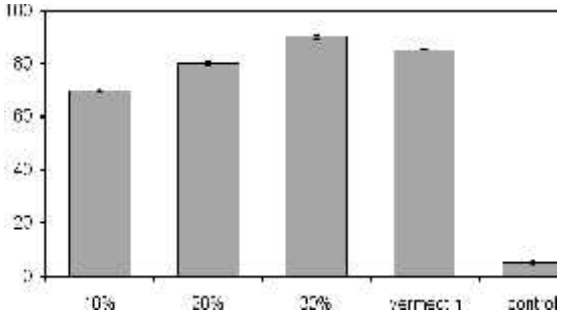

Figure I. Miticidal activity of *Vitex negundo* (10, 20 and 30%) extract and that of the ivermectin against the *Sarcoptes scabiei* in comparison with the control treatment using *in vitro* laboratory test after 28 days. The bars on the charts showing two standard errors (±) calculated on three replicates of each treatment.

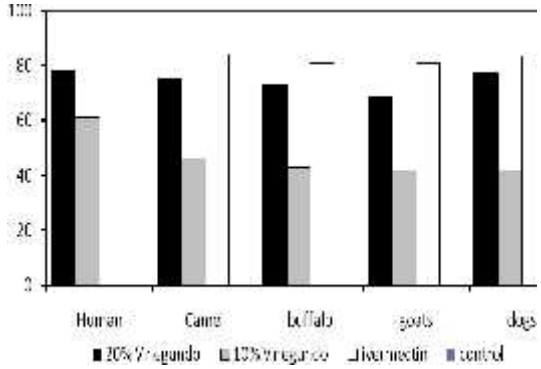

Figure II. Miticidal activity of *Vitex negundo* (10 and 20%) extract and ivermectin against the *Sarcoptes scabiei* mites in comparison with the control using *in vivo* laboratory *test after* 5th week of treatment.

TABLE-I t-Test: Two-Sample Assuming Equal Variances with (10, 20 and 30%) *V.negundo* concentration verses Ivermectin and Pure methanol in vitro (Laboratory test)

	30% v. negundo extract	*20% v. negundo extract*	*30%v.negundo extract*	*10% Vitex negundo extract*	*30%V negundo extract*	*Ivermectin) 10mg/ml*	*30%V. negundo extract*	*Methanol*
Mean	18.4	16.8	18.4	15.2	18.4	17.6	18.4	5.6
Variance	1.3	3.7	1.3	7.7	1.3	2.3	1.3	65.3
Observations	5	5	5	5	5	5	5	5
Pooled Variance	2.5		4.5		1.8		33.3	
Hypothesized Mean Difference	0		0		0		0	
Df	8		8		8		8	
t Stat	1.6		2.385		0.942		3.507	
P(T<=t) one-tail	0.074N.s		0.0220*		0.186 N.s		0.003***	
t Critical one-tail	1.859		1.859		1.859		1.859	
P(T<=t) two-tail	0.148N.s		0.044f*		0.373 N.s		0.007**	
t Critical two-tail	2.306		2.306		2.306		2.306	

TABLE-II: t-Test: Two-Sample Assuming Equal Variances (interval reading in topical use)

	1st week	2nd week	1st week	4th week	1st week	5th week
Mean	14.533	25.0333	14.533	37.533	14.533	55.366
Variance	70.746	156.006	70.746	390.506	70.746	771.046
Observations	6	6	6	6	6	6
Pooled Variance	113.376		230.626		420.896	
Hypothesized Mean Difference	0		0		0	
Df	10		10		10	
t Stat	-1.708		-2.623		-3.447	
P(T<=t) one-tail	0.059 N.s		0.012*		0.003***	
t Critical one-tail	1.812		1.812		1.812	
P(T<=t) two-tail	0.118 N.s		0.025*		0.006**	
t Critical two-tail	2.228		2.228		2.228	

TABLE III: t-Test: Two-Sample Assuming Equal Variances (among Human and different mentioned animals in topical use).

	25	*Camel*	*25*	*Buffalo*	*25*	*Goat*	*25*	*Dog*
Mean	46.428	35.714	46.428	30.625	46.428	28.125	46.428	34.125
Variance	470.285	320.238	470.285	322.267	470.285	302.982	470.285	301.267
Observations	7	7	7	8	7	8	7	8
Pooled Variance	395.261		390.583		380.199		379.276	
Hypothesized Mean Difference	0		0		0		0	
Df	12		13		13		13	
t Stat	1.008		1.545		1.813		1.220	
P(T<=t) one-tail	0.166NS		0.073NS		0.046*		0.121N.s	
t Critical one-tail	1.782		1.770		1.770		1.770	
P(T<=t) two-tail	0.333 N.s		0.146 N.s		0.092 N.s		0.243N.s	
t Critical two-tail	2.178		2.160		2.160		2.160	

DISCUSSION

Scabies is a skin disease caused by mite (*Sarcoptes Scabiei*). The entire life cycle of the *Sarcoptes scabiei* mite completes on its hosts skins surface in 17-21 days (Soulsby, 1982). The disease is highly contagious and intensely pruritic characterized by crust formation and is transmitted by direct contact to the effected host. It can affect people as well as animal health and reduce their normal growth. In severe circumstances of scabies, the hosts may terminate fatally. Due to resistant strains of *Sarcoptes scabiei* mite and adverse effects of some medicines upon the curing persons or animals, have made scabies a challenging disease to be diagnosed and properly cured (Anderson, 1982). Topical use of *Vitex negundo* extract showed a high miticidal activity against *Sarcoptes scabiei* on peoples and different animal species. This indicates that methanolic extracts from *Vitex negundo* may possess effective miticides against the mites on people and animals. Modern miticides are effective but most of them possess adverse effects on the animals and persons receiving treatment such as ivermectin use in Norwegian scabies, when repeatedly treated showed resistance (Currie *et al.,* 2004). Ivermectin, a running parasiticide available in the market (at the rate of 0.2 mg/Kg live body weight of affected animals has shown 70% (Campbell, 1981), 80% (Burgos and Huici 1984) and 90% (Maqbool *et al*., 1992) protection against Sarcoptes scabiei infestation. While in the present study, ivermectin showed 85% and 30% *Vitex negundo* showed 90% miticidal activity. *Vitex negundo*-Lam. methanolic extract is easily prepared, have no drug resistance producing factors and have no tissue residual impact. According to TLC analysis *Vitex negundo*-Lam. has alkaloids, saponin and flavonoids. *Vitex negundo*-Lam.plant has diversified nature containing *vitexicarpins* as an active compound which has flavonoids analogous structure with cytotoxic effect as well as β–Caryophyllene and α-pinine having acaricidal and insecticidal effect (Sahare *et al.,* 2008 and Liu et *al*., 2010). Apart from well documented antioxidant role, flavonoids behave as pro oxidants. Hence it appears that flavonoids in this plant extract might be responsible for miticidal effect as it has shown antifilarial activity. (Sahare *et al.,* 2008). Hence it is suspected that the flavonoids, β-caryophyllene and the α-pinine present in the extracts of *Vitex negundo* might be the reason of its

miticidal effect against the *Sarcoptes scabiei* mites as has been reported previously for their antifilarial, insecticidal and insect repellent activities (Sahare *et al.,* 2008 and Liu et *al*., 2010). Although the present study has shown that *Vitex negundo* methanolic extract could be effective against the *Sarcoptes scabiei* mites induced disease (scabies), however, further studies may be useful to explore the specific bioactive compounds responsible for its bio-medicinal impacts upon the scabies disease in people and animals.

Conclusions: It is concluded from the therapeutic and laboratory trials that beside other synthetic medicinal agents such as lindane, permethrin and ivermectin available in the market for the control of ectoparasits, the methanolic extract of *Vitex negundo* may be used both *in vitro* and *in vivo*, and could be useful to be applied as an effective bio-medicine for the treatment of scabies in people and animals skins, however further research for *Vitex negundo* detail compound analysis and the trials for their candidature as miticide may be needed to more deeply exploit the medicinal efficacy of the *Vitex negundo* against scabies disease.

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