Anti-Oxidant, Pediculicidal And Pesticidal Activities Of Leaves Of *Rhinacanthus Nasutus* (Linn) Grown In Sri Lanka

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Abstract

Objective:*Rhinacanthus nasutus* is an ethnomedical plant used in the traditional system of medicine for treating skin diseases. As no adequate studies available of leaves of *R. nasutus* grown in Sri Lanka, this study aimed on exploring anti-oxidant, anti-parasitic and pesticidal activity of leaves extract of the plant.
Method: Antioxidant capacity of the crude extract of leaves was evaluated using DPPH free radical scavenging assay and FRAP assay using ascorbic acid and $FeSO_4.7H_2O$ as the standards respectively. Pediculicidal efficacy was carried out against head lice using 0.0010% (w/v) methanolic, ethyl acetate and aqueous crude extracts of leaves by following modified filter paper diffusion bioassay. Pesticidal activity of leaves was evaluated using modified filter paper contact bioassay against mealybugs, *P. marginatus,* resides on papaw and guava trees.

Results: The IC_{50} value of DPPH assay was found to be 514.3 μgmL^{-1} and FRAP value was found to be 594.33±2.93 µmol Fe^{2+}/g in anti-oxidant assay. The LT_{50} (median Lethal Time) values for 0.0010% (w/v) concentration of methanolic, ethyl acetate and aqueous crude extracts were 263, 347 and 676 min respectively in pediculicidal assay and LC_{50}(50% mortality) value was found to be 0.0011% (w/v)after 240 minutes where as LT_{50} values of 0.0020% (w/v) of methanol, ethyl acetate and aqueous fractions of *R. nasutus* for adult were found as 31, 124 and 189 hoursand for nymph were 14, 18 and 29 hours respectively in pesticidal assay.Accordingly, the methanolic extract was effective in controlling nymph stage of mealybug life cycle than adultswith the LC_{50}of0.0015% (w/v) within 14 hours.

Conclusion: This study confirmed the promising pediculicidal and pesticidal activity and significant anti-oxidant capacity ofleaves of *R. nasutus.*

Keywords- *R. nasutus,* pediculicidal activity, pesticidal activity, DPPH assay, FRAP assay

INTRODUCTION

Plants and plant based products have been used for medicinal and health enhancement purpose since human civilizations and indeed since the evolutionary origin of humans. With the development of science, focus on plant research has increased all over the world andthe research on plants that have been used in traditionally for various healthcare purposes have been intensified with the purpose of finding novel bioactive material and potential applications as greener value-added products [1]. Vast structural diversity of secondary metabolites presents in plants have been recognized as the prime important factors for diverse biological activities exerted by them [2]. Plant anti-oxidants such as flavonoids, polyphenols are known to involve in quenching harmful free radicals generated in the cells resulting the reduction of excessive oxidative stress in the cell that has been known to associate with reduction of the risk of many non-communicable diseases (NCD's) [3-5].

Currently, the secondary factors such as food habits and use of synthetic chemicals in agriculture have been identified to make direct correlation with prevalent NCD's [6]. Therefore, there is an urgent need to shift into plant based material to replace synthetic chemicals used as

pesticides, herbicides etc. and to find safer alternative fast foods with the incorporation of natural potential compounds such as anti-oxidants. The plant, *Rhinacanthusnasutus* (Acanthaceae), commonly known as "HeenAniththa" in Sri Lanka, is an ethnomedical plant which is widely distributed in North Central and Southern provinces in Sri Lanka. The history has noted the use of this plant in Sri Lankan traditional system of medicine to treat various ailments including eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension and several skin diseases [7]. Some bioactive compounds and some pharmacological activities have been discovered from leaves of *R. nasutus* by researchers around the globe [7-14]. The main bioactive constituents identified in the leaves were naphthoquinonesand rhinacanthones which are known to have anti-proliferative activity against a panel of cancer cells. In the contest on Sri Lanka, no reports are available on investigation of this plants, and so farno studies were done to explore its pediculicidal and pesticidal activities. Therefore, this study was aimed on investigation of anti-oxidant capacity, pediculicidal and pesticidal activities of leaves of *R. nasutus* grown in Sri Lanka

MATERIALS AND METHODS

Chemicals used in the study:

All the chemicals and reagents including methanol, hexane, ethyl acetate, chloroform, L-ascorbic acid, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine(TPTZ) used in this study were of analytical grade and purchasedfrom Sigma-Aldrich.

Plant Material:

Leaves of *R. nasutus* were collected from a domestic cultivation of Puttalam district, Sri Lanka and authenicated. Healthy leaves were washed and dried under breeze at room temperature for one week. The dried leaves were ground to fine powder using a blender and were stored in sealed containers at 4 °C until usage.

Extraction of plant constituents

The methanolic extract of leaves was prepared by maceratingin methanol for five days with frequent agitation. The macerated solution was filtered and concentrated under vacuums, and suspended in distilled water. The water suspended materialwas sequentially extracted with n-hexane, chloroform and ethyl acetate, and combined fractions were concentrated under vacuum and remaining fraction was used as aqueous extract [15]. All fractions were stored at 4 °C until tests were performed.

Determination of antioxidant capacity

Anti-oxidant capacity was determined using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH assay) and ferric reducing antioxidant power assay (FRAP assay).

DPPH radical scavenging assay

The free radical scavenging activity of the plant extract against DPPH radical was determined by the slightly modified DPPH method[16]. The concentrations of plant extractswere preparedas 25, 50, 75, 100, 200, 400, 600, 800 and 1000 μgmL^{-} Ascorbic acid was used as the standard, and the similar concentrations were prepared with the plant extract.The test solutions were prepared by mixing 1 mL of the extract and standard with 3 mL of DPPH solution. The solutions were kept in dark for 30 minutes in room temperature. The absorbance was measured at 517 nm using the UV-Vis spectrophotometer (UH5300 -HITACHI). The free radical scavenging activity was expressed as IC_{50} using % inhibition vs. concentration plot. The % inhibition was calculated from[(AB-AA)/AB] x100, Where AB is the absorption of the DPPH without extract and AA is the absorption of the DPPH solution containing plant extract or ascorbic acid.

FRAP assay

The total antioxidant power of plant extract was determined using a slightly modified FRAP assay reported by Gohari*et al.*[17]. Freshly prepared FRAP reagent (3.00 mL) was mixed with 0.4 mL of extract and absorbance was recorded at 593 nm after 30 min incubation at 37ºC. An aqueous

solution of $FeSO_4.7H_2O$ (20-150 μgmL^{-1}) was used as the standard for calibration.

Pediculicidal assay

Adult head lice were collected from infested children 7-15 years old. The lice collection protocol was conducted after approval of their parents. The lice were collected by visual search of the hair and combing the scalp using a fine-toothed anti-louse comb. Viable head lice were obtained and pooled by removing them from the comb into clean glass boxes and kept cool and shaded [18].

Modified filter paper diffusion bio assay was used to evaluate pediculicidal activity of plant extracts [19].*In-vitro*test was started within an hour after collection of head lice. Filter paper discs and strips coinciding with internal surfaces of petri dish were placed and lice were kept on filter papers and were exposed to fixed concentration 0.0010% (w/v) of different extracts*R. nasutus*, which was prepared dissolvingcrude extracts in water using sonication.Then the petri dishes were covered with the cellophanes with small holes allowing ventilation. Distilled water was used as negative control and commercially availablehead lice lotion containing 0.5%(w/v) permethrin was used as positive control. Lice mortalities were determined in specific time intervals at 0.5, 1, 2 ,4 and 8 hours after treatment. The mortalities of head lice on the filter paper were assessed using magnifying lens. The criteria used to evaluate survival of lice were defined as the complete absence of any vital signs. If any signs of life such as leg movements were observed, the lice were considered as alive. The lice were judged as dead if there were no vital signs at all. The mortality was calculated using the following formula [20].

$$\text{Mortality} = \frac{Total\ number\ of\ dead\ lice}{Total\ sample\ size} \times 100$$

The time which kill a 50% of head lice (LT_{50}) were determined varying the concentrations [21].

Pesticidal assay

Mealybugs were collected as pests from infested plants of papaw and guava cultivation which is located in the University of Ruhuna, Sri Lanka premises. They were taxonomically identified as *P. marginatus* with the help of Department of Zoology, University of Ruhuna, Sri Lanka. Then adult mealybug individuals and nymphs were separated in to glass box using a tiny brush [22].

Filter paper contact bioassay with slight modifications was conducted to evaluate thepesticidal activity[23]. The setup of the experiment was same as the above pediculicidal assay. Two life stages of mealybugs such as nymphs and adult females were tested in this assay. Distilled water was used as negative control. Pest mortality was recorded in specific time intervals at 2, 4, 6, 12 and 24 hours after application. The mealybugs were judged as dead if there were no body movements at all. Then the median lethal time (LT_{50}) and lethal concentration (LC50) was calculated as above using probit analysis.

Statistical Analysis

Mortality data of head lice and mealybugs at all treatments were subjected to probit analysis to determine the LT_{50} and LC_{50} values of each solvent extracts of *R. nasutus*. R statistical software was used for all these statistical analyses.

RESULTS

Anti-oxidant activity

Methanolic extract of *R. nasutus* was used to determine the anti-oxidant activity by DPPH radical scavenging assay using ascorbic acid as a standard.The results of percentage inhibitions of standard and methanolic extract with respect to the concentrations are given in the Figure 1.

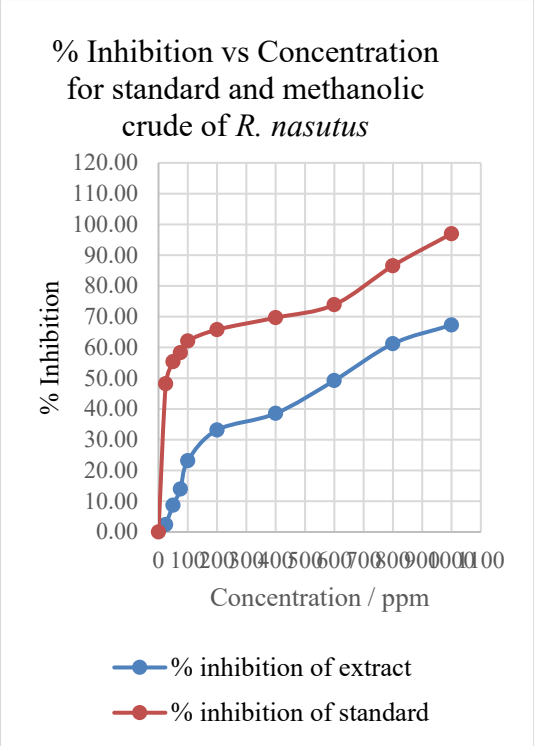

Figure 1: Percentage inhibition versus concentration plot for the standard series and the extract series in DPPH assay

According to the percentage inhibition of DPPH assay, the calculated IC_{50} values were 40.7µgmL^{-1} and 514.3 µgmL^{-1}for ascorbic acid and methanolic extract of *R. nasutus* respectively.

In FRAP assay, the antioxidant capacity was determined using the reducing power of the plant extract.Absorbance values are obtained from triplicate of FRAP experiment.The mean FRAP equivalent value was found to be 594.33±2.93 µmol Fe^{2+} /g(crude).

Pediculicidal Activity

The average percentage mortality of head lice in methanol, ethyl acetate and aqueous extracts of *R. nasutus*is shown in Table 2.

Table 2: Average percentage mortalities of head lice to different plant extracts of *R. nasutus*

Time /min	Average % mortality				
	Methanol	Ethyl acetate	Aqueous	Negative control	Positive control
30	0	0	0	0	90
60	0	0	0	0	100
120	10	10	0	0	100
240	50	30	10	0	100
480	90	60	40	0	100

LT_{50} values obtained from probit analysis for mortality values of 0.0010% (w/v) of methanolic, ethyl acetate and aqueous extract were found to be 263, 347and 676 min. The methanolic fraction were highly active against head lice.

The whole bioassay procedure was repeated for active methanolic extract of *R. nasutus* with varying concentrations as 0.0005%, 0.0010%, 0.0015%, 0.0020% and 0.0025% in w/v basis. LC_{50}(50% mortality) value was found to be 0.0011% (w/v) after 240 min which was calculated using the graph of probit mortality versus log concentrationas indicated in the Figure 2.

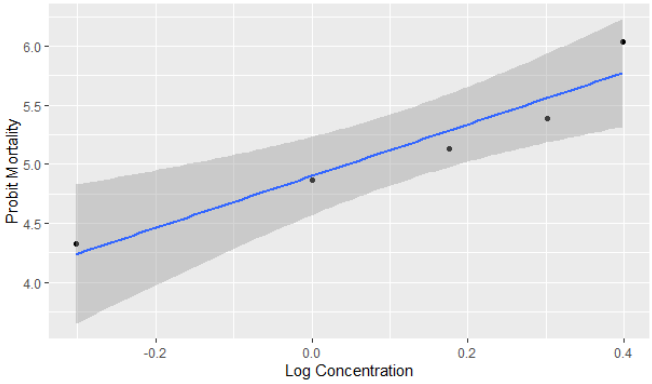

Figure 2: The graph of Probit mortality vs. Log concentrationin Pediculicidal Assay

Pesticidal assay

The average percentage mortality values for adults and nymph stages of mealybug is given in the Table 2.

Table 2: Average percentage mortalities of adult mealybugs and nymphs to different *R.nasutus* extracts with respect to different exposure times.

Time / hours	Average percentage mortality / %					
	Adult mealybugs			Nymphs of mealybugs		
	Methanol	Ethyl acetate	Aqueous	Methanol	Ethyl acetate	Aqueous
2	0	0	0	0	0	0
4	0	0	0	0	0	0
6	0	0	0	15	5	0
12	10	0	0	60	35	10
24	35	20	10	85	60	45

Based on the results different extracts of *R. nasutus*it shows the significantnymphal mortality than the adult mealybug mortality.LT_{50} values of each 0.0020% (w/v) of methanolic, ethyl acetate and aqueous factions were 31, 124 and 189 hours for the adult mortality and 14, 18 and 29 hours for the nymph mortality respectively. Methanolic fraction was highly active against both adult and nymph stage of the mealybug. LC_{50}(50% mortality) value was 0.0015% (w/v) after 14 hours which was calculated by using the graph of probit mortality versus log concentration as indicated in the Figure 3.

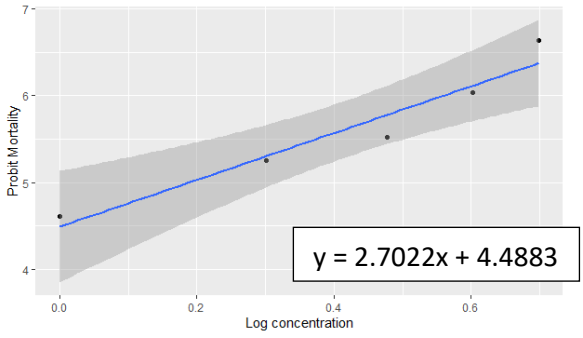

Figure 3: The graph of Probit mortality vs. Log concentrationin Pesticidal assay

DISCUSSION

Anti-oxidant activity

Based on the results ofDPPH radical scavenging assay, it shows thatmethanolic extract possesses significant radical scavenging activity (514.3 μgmL^{-1}). DPPH is a stable radical which is reduced in the presence of hydrogen donating antioxidants. Polyphenols present in plants are main responsible for anti-oxidants activity exerted. When comparing the above result with previously recorded literature, Sasikumar *et al.* has reported the IC_{50} value of 230 $\mu gmL^{-1}$for dry powder of the plant[24]. Bukke*et al.* has also reported the antioxidant power of hexane, ethyl acetate, methanol and water extracts of *R. nasutus* leaves using DPPH assay, the values of 50% inhibition level are lower than values obtained in this study [25]. The percentage inhibition for the highest concentration (1000 ppm) of the methanol extract in previous study was 35% and whereas our study gave it as 67.28%.

The FRAP assay treats the antioxidant in the sample as reductants in redox linked colorimetric reaction and the value reflect the reducing power of the antioxidants. Here antioxidants react with Fe^{3+} -TPTZ to produce a coloured Fe^{2+}-TPTZ complex which is measured at 593 nm. FRAF value of this study showed the highest FRAP value indicating highest total antioxidant capacity. Notably, there are no available records from Sri Lankan studies to compare the antioxidant capacity of*R. nasutus* grown in Sri Lanka.

Pediculicidal assay

The data in this bioassayhighlights thatmethanolic extract of *R. nasutus*ishighly toxic to head lice with 90% mortality at 480 min. Further, the 0.0010% (w/v) of methanolic extract has lowest LT_{50} value. Therefore, methanol extract showed high levels of mortality on adult lice in short time than the other extracts. And the calculated LC_{50} value for methanolic extract was 0.0011% (w/v) after 240 min. Based on the above results, it is evident that *R. nasutus* has high potential to kill lice and hence, therefore there is an extensive

scope for development into products of pediculicidal activity.It is noteworthy that there is no recorded literature available for this plant species in terms of pediculicidal studies and this work is recognized as the first study related to this.

Pesticidal activity

The data in this pesticidal assaybrought to notice that nymph stage of mealybug life cycle is the most susceptible stage to the plant extracts in term of pesticidal activity, and methanol extract showed 50% of mortality on nymph stage of mealybugs in 14 hours, with LC_{50} value of 0.0015% (w/v). It is noteworthy that there is no recorded literature available for this plant species in terms of pesticidal activity against mealybugs. However, *R. nasutus* has been studied against few another pest insect [26 -27].In this study, the pesticidal activity was evaluated after 24 hours and 50% mortality of nymphs could be achieved within 14 hours, which gives a promising method that applicable to local use. However, contact pesticides are less effective against mealybugs because of their cryptic habitats in plants and the water proof waxy layer on the body[28].Therefore, the field trials should be necessary in order to check the reliability of plant extracts in control of mealybugs. Also,these results gives an insight into the potential use of the *R. nasutus* as botanical pesticide especially against mealybug species on cultivated fruit crops in Sri Lanka.

CONCLUSION

In summary, we have successfully demonstrated for the first time that the leaves of *R. nasutus*exert promising pediculicidal activity and pesticidal activity against mealybugs.It was noted that methanolic extract of *R. nasutus* had the highest activity in all performed tests.Further this study confirm the significant antioxidant properties of the methanolic extract as well, which signifies the multifunctional therapeutic potential of *R. nasutus*. Further clinical trials and field trials will be needed to assess the accuracy of the laboratory results of the*in-vitro* pediculicidal and pesticidal assays respectively in order to develop it intovalue added products.

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