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RESEARCH ARTICLE

In vitro Efficacy of *Areca catechu* against Cypermethrin Resistant *Rhipicephalus microplus* and its Phytochemical Analysis

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A B S T R A C T

This study investigated the use of the crude extract alongwith, water and petroleum ether fractions of *Areca* (*A.*) *catechu* seeds against cypermethrin resistant *Rhipicephalus* (*R.*) *microplus*. A colony of cypermethrin-resistant *R. microplus* was maintained on cattle calves. *Areca catechu* seeds were purchased from the local market of Faisalabad. The crude aqueous methanol extract (CAME) and two fractions were prepared using standard phytochemical procedures. All these extracts/fractions were used to check their efficacy against cypermethrin-resistant *R. microplus* by using the syringe test (Modified Larval Immersion Test). Mortality (%) results obtained in the syringe test were subjected to the Probit (dose-response) analysis and phytochemical analysis was performed to check the presence of active group of compounds. Crude extract and two fractions of *A. catechu* were found to be effective against cypermethrin resistant *R. microplus*. The most effective fraction of *A. catechu* was petroleum ether fraction with LC_{50} = 0.3, LC_{90} = 0.8 and LC_{99} = 1.7. Results of Petroleum ether fraction were significantly different ($p < 0.05$) from other two tested fractions. In the phytochemical analysis carbohydrates, alkaloids, flavonoids, tannins, phenols, glycosides, volatile oils and saponins were found to be the group of compounds. These results indicated that *A. catechu* has acaricidal potential against cypermethrin-resistant *R. microplus*.

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INTRODUCTION

Ticks are obligate blood sucking parasites that infest a wide range of hosts with a worldwide distribution. While considering livestock enterprises, 80% population of cattle worldwide is affected by ticks and tick-borne diseases (TTBDs) (Ghosh *et al*., 2006; Karim *et al*., 2017; Shaukat *et al*., 2019; Ghafar *et al*., 2020). It has been estimated that TTBDs result in global economic losses of 20-30 billion US$ per anum (Lew-Tabor and Rodriguez Valle, 2016). Among different species, cattle tick, *Rhipicephalus* (*R.*) *microplus* is considered to be of great significance because of its distribution in the tropical and subtropical regions of the world including South and Central America, India, Australia, China and Malaysia where it is present for decades (Labruna *et al*., 2009). Economic losses caused by *R. microplus* were estimated to be 2 billion US$ in Brazil and 62 million US$ in Australia (Rodrigues and Leite, 2013).

Cypermethrin (synthetic pyrethroid) has been found to be the most abundantly used acaricide against ticks. The intensive use of acaricides has resulted in the accumulation of drug residues in milk and meat along with the emergence of multidrug resistance (Klafke *et al*., 2017). Since there are various reports of the development of acaricide resistance from different parts of the world, some alternate management practices are being used for the control of ticks such as vaccines, grooming, pasture management, biological control, genetic manipulation, endosymbiotic approach and medicinal plants. Plant-based treatment is the principal alternate among all these because their efficacy is known for thousands of years (Wang and Li, 2005; Moryani *et al.,* 2021). There is a long history of use of medicinal plants to control the ticks particularly in traditional cultures of Asia and Africa by resource-poor farmers. The scientific community, therefore, has an increasing interest to find out the new potential compounds which are effective against *R. microplus*. It has been documented by many researchers that plant extracts have great potential for the control of various stages of ticks (Ghosh *et al*., 2011; Sindhu *et al*., 2012; Abbas *et al*., 2018; Salman *et al*., 2020; Zaheer *et*

al., 2021). Application of biopesticides has shown promising results against ticks, especially *R. microplus* (Kamaraj *et al.,* 2010; Ghosh *et al.,* 2011), however, the knowledge about their efficacy against cypermethrin resistant *R. microplus* is scarce.

Various medicinal plants are well known for their antiparasitic activity (Abbas *et al*., 2017; Fayaz *et al*., 2019; Abbas *et al*., 2020; Hussain *et al*., 2021). *Areca* (*A.*) *catechu* belonging to the family Arecaceae, is more prevalent in Southeast and Southern Asian countries including New Guinea, China, Malaysia, India, Indonesia and the Philippines. It is commonly known as areca nut. The fruit of this plant is used as a chewable item and also has traditional value as herbal medicine (Heatubun *et al*., 2012). Over 400 million people chew it daily in tropical regions including South Asia and China. This plant has many pharmaceutical properties including anti-inflammatory, anti-oxidant, anti-allergic, anti-bacterial, anti-analgesic and anti-parasitic. Almost 59 compounds have been isolated from this plant, which includes tannins, alkaloids, fatty acids, flavonoids and others (Liu *et al*., 2013). It is described as a great herbal medicine to promote digestion and also used to kill parasites. *Areca catechu* has proven efficacy against parasitic diseases, especially acts as an anti-malarial agent (Boniface *et al*., 2014).

Although *A. catechu* has huge acaricidal potential (Bigg and Purvis, 1976; Zaman *et al*., 2017), its efficacy against cypermethrin-resistant *R. microplus* has not been reported yet. Thus, this study was designed to check the acaricidal efficacy of *A. catechu* and its various fractions against cypermethrin-resistant *R. microplus* by using a syringe test. Phytochemical analysis of the crude extract and various fractions of *A. catechu* was also done to ascertain the active group of compounds.

MATERIALS AND METHODS

Maintenance of resistant tick colony: Engorged female *R. microplus* ticks were collected from District Okara, Punjab, Pakistan. Cypermethrin resistance was diagnosed by using the larval packet test (LPT) recommended by FAO (Kemp *et al*., 1999). After the diagnosis of cypermethrin resistance (submitted manuscript), a tick colony was maintained on calves by feeding the larvae of cypermethrin-resistant *R. microplus*.

Processing of plant materials: Seeds of *Areca catechu* **were** purchased from the local market of Faisalabad. The CAME of *A. catechu* (seeds) was prepared by the method of Tabassam *et al*. (2008). Briefly, powdered dried plant material was soaked in an aqueous-methanol (30:70) solvent for approximately 36 hours. Subsequently, filtration of the soaked material was done with four layers of muslin cloth and the fresh solvent was added to the same material. This process was repeated three times and the combined filtrate was evaporated in a rotary evaporator under reduced pressure to obtain the CAME. Complete drying was done in the freeze dryer.

Fractionation of CAME and identification of active group of compounds: Different fractions (petroleum ether, ethyl acetate, chloroform, methanol and water) of CAME were prepared by using standard phytochemical procedures (Williamson *et al*., 1998). First of all, CAME was dissolved in an appropriate quantity of petroleum ether. Extract dissolved in petroleum ether was filtered and more petroleum ether was added and the same procedure was repeated until petroleum ether became transparent. All these filtrates were combined and condensed in the rotary evaporator (Heidolph, Heidolph instruments, GmbH & Co. KG Walpersdorfer Str. 12 D-91126 Schwabach, Germany) and then condensate was freeze dried. Likewise, other fractions were prepared from the remaining CAME. All the dried extracts were stored at 4 °C until used. Qualitative analysis of these fractions was performed to check the active group of compounds like carbohydrates (Sofowara, 1993), alkaloids (Evans, 1997), flavonoids (Raman, 2006), tannins (Trease and Evans, 1989), phenols (Mace Gorbach, 1963), glycosides (Sofowara, 1984), volatile oils (Trease and Evans, 1989), fixed oils and fats (Sofowara, 1993; Tiwari *et al*., 2011) and saponins (Kumar *et al*., 2009).

***In vitro* evaluation of acaricidal activity:** For all *in vitro* trials with crude extract and its fractions, syringe test (Sindhu *et al*., 2012) was used. Briefly, a 3 ml syringe was cut from its nozzle end and about 200 (10 mg) eggs were put in these syringes. Nylon gauze was used to seal the open end of these syringes, which were placed in the incubator under conditions of 27°C and 85-90% relative humidity (RH) to obtain 14-day old larvae. To prepare the stock solution, CAME and other fractions were dissolved in 0.2% solution of TritonX-100 and subsequently, five serial dilutions of the stock solution were prepared. For treatment, 2 ml test solution was drawn into the syringe and shaken for 30 seconds to treat the 14-day-old larvae. Treated syringes were put in a fume hood for two hours to dry. Then these syringes were placed in the incubator and number of live ad dead larvae was counted after 24 hours.

Statistical analysis: Data obtained in the form of percent mortality were subjected to probit (dose-response) analysis by using the Polo-Plus software (LeOra Software, 2002) for estimation of lethal concentrations (LC_{50}, LC_{90} and LC_{99}), their respective 95% confidence limits and the slope.

RESULTS

Most of the values of standardized residuals were found to be between -2 and 2, indicating the results in a good fit of the probit model (Fig. 1). Probit mortality x log concentration plot and standard residuals of data from *R. microplus* submitted to syringe test with different fractions of *A. catechu* (seeds) have been shown in Fig. 1.

Based on $LC_{50,}$ LC_{90} and $LC_{99,}$ the most effective fraction of *A. catechu* was petroleum ether fraction with LC_{50} = 0.3, LC_{90} = 0.8 and LC_{99} = 1.7 as compared to other products tested. A statistically significant ($P < 0.05$) difference was observed between LC_{50}, LC_{90} and LC_{99} values of petroleum ether fraction. Similarly, The LC_{50} values of CAME, petroleum ether and water fractions were observed to be 1.12, 0.3 and 10.1 respectively, which showed a statistically significant difference ($P<0.05$) from each other. The relationship between

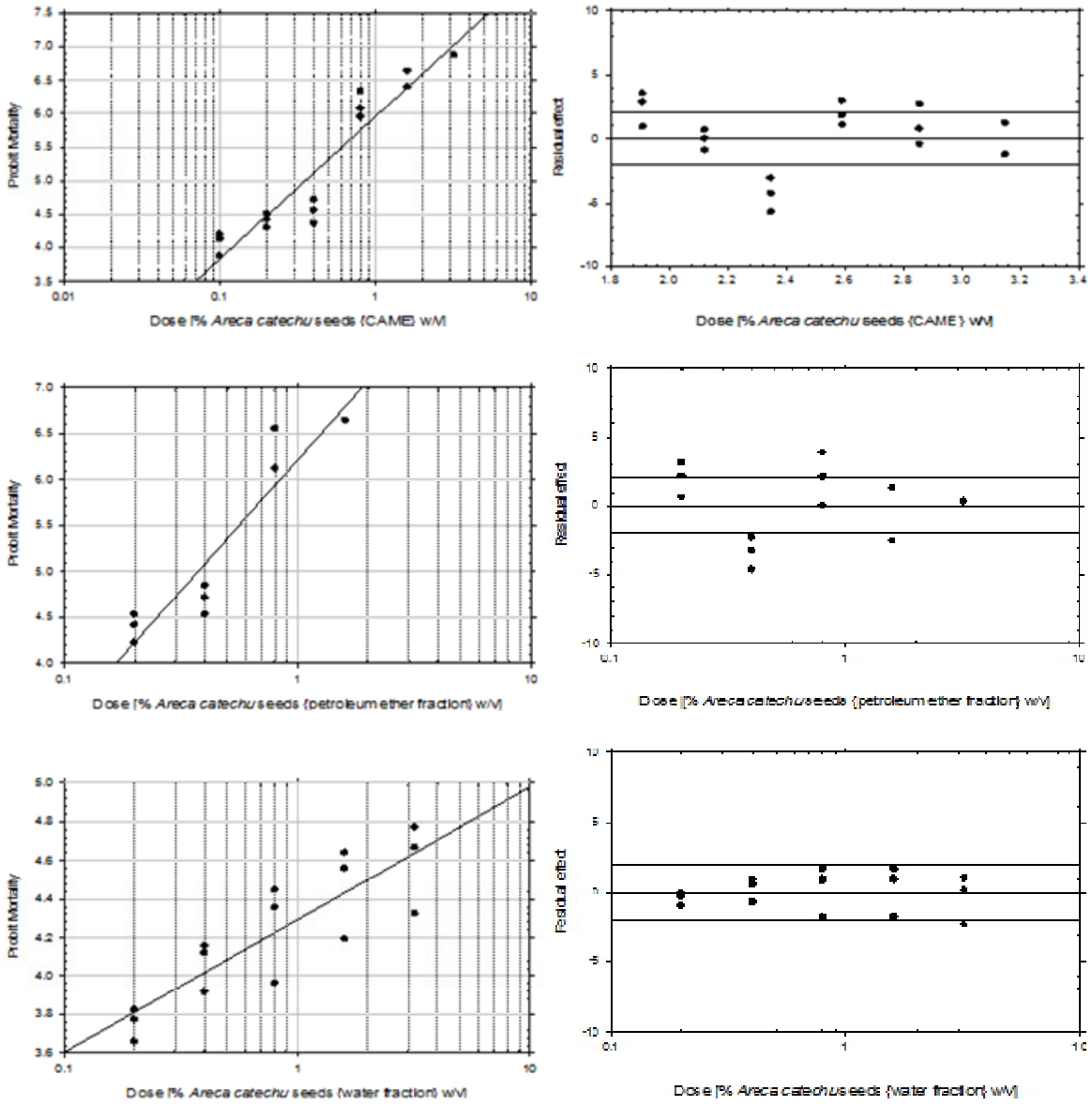

Fig. 1: Graphical representation of probit analysis data obtained after treating 14 days old *Rhipicephalus microplus* larvae with serial dilutions of crude aqueous methanol extract, petroleum ether fraction and water fraction of *Areca catechu* (seeds)

mortality percentages against exposure concentrations was checked by using linear regression and a strong positive relationship was observed between probit mortality and different concentrations of *A. catechu* fractions with chi-square values of 109.03, 82.14 and 22.0 for CAME, petroleum ether and water fractions, respectively (Table 1). The results indicated a concentration-dependent increase in mortality data tested with all three fractions. Among three candidate drugs, water fraction was found to be the least effective with LC_{50} = 10.1, LC_{90} = 795.2 and LC_{99} = 27965.9. The results of water fraction showed a non-significant ($P > 0.05$) difference between LC_{90} and LC_{99} values. Similarly, the LC_{50}, LC_{90} and LC_{99} values of CAME were found to be 1.12, 4.1 and 11.7 respectively, which were statistically different ($P < 0.05$) from each other.

Phytochemical analysis was done to find the presence of secondary metabolites like carbohydrates, alkaloids, flavonoids, tannins, phenols, glycosides, volatile oils, fixed oils and saponins. Crude extract was containing carbohydrates, alkaloids, flavonoids, tannins, phenols, glycosides, volatile oils and saponins. Fixed oils and fats were not found in any fraction. Carbohydrates, phenols, glycosides, volatile oils and saponins were found in all the fractions of *A. catechu*. Furthermore, different fractions of the same plant extract showed the presence of different groups of compounds. Qualitative analysis of the crude extract and two fractions of *A. catechu* has been shown in Table 2.

DISCUSSION

An important indication about the good fit of results in log probit model is the standardized residuals value (Robertson *et al*., 2002). If the values of standardized residuals fall within a horizontal band near-zero (usually between -2 and 2), it is considered to be a good fit in this model. In the present study, most of the values of

Table 1: Comparison of the LC_{50}, LC_{90} and LC_{99} estimates of different extracts/fractions of *Areca catechu* against cypermethrin resistant population of *Rhipicephalus microplus*, *in vitro*

Sr. No.	Name of extract/fraction	Slope (SE)	X^2	LC_{50} (95% CL)	LC_{90} (95% CL)	LC_{99} (95% CL)
1	CAME	2.3 (0.1)	109.03	1.12 (0.9-1.4)	4.1 (3.1-6.2)	11.7 (7.5-23.0)
2	Petroleum ether	3.3 (0.2)	82.14	0.3 (0.3-0.4)	0.8 (0.7-1.2)	1.7 (1.2-3.1)
3	Water	0.7 (0.1)	22.0	10.1 (4.9-43.4)	795.2 (126.4-39729.7)	27965.9 (1735.6-10580826.7)

Table 2: Qualitative analysis crude extract and different fractions of *Areca catechu*

	Phytochemicals								
Plant fraction	Carbohydrates	Alkaloids	Flavanoids	Tannins	Phenols	Glycosides	Volatile oils	Fixed oils and fats	Saponins
Areca catechu CAME	+	+	+	+	+	+	+	-	+
Areca catechu petroleum ether fraction	+	-	-	-	+	+	+	-	+
Areca catechu water fraction	+	-	+	+	+	+	+	-	+

standardized residuals were found to be between -2 and 2, indicating the results in good fit of the probit model (Fig. 1).

The therapeutic efficacy of medicinal plants is due to the presence of bioactive compounds (Mustafa *et al*., 2017). Phytoconstituents in the plants have the potential to be used in the development of acaricides (Afrin *et al*., 2018). Current knowledge about the efficacy of plant extracts as acaricides was reviewed by Benelli *et al*. (2016) with special emphasis to *R. microplus, R. appendiculatus, R. turanicus, R. sangueneus, Ixodes* (*I.*) *persulcatus, I. ricinus, Haemaphysalis* (*H.*) *longicornis, H. bispinosa, Amblyomma cajennense, Hyalomma* (*H.*) *marginatum rufipes, H. anatolicum,* and *Ranunculus pulchellus*.

Nature serves as the storehouse of various active ingredients which are used in modern pharmaceuticals today. Many secondary metabolites are synthesized by the plants which help them to reproduce and survive. Recent studies have focused on the determination of active ingredients in medicinal plants with an increasing trend in their preparations. The reason behind this trend is that there are many secondary metabolites in these plants with a wide range of biological activities for their use in various medicines (Buchanan *et al*., 2015). These secondary metabolites are carbohydrates, alkaloids, flavonoids, tannins, phenols, glycosides, volatile oils, saponins, phytoalexins, terpenoids and steroids, etc. as found in this study.

In the present study, chloroform, ethyl acetate and methanol fractions of *A. catechu* could not be dissolved which is in accordance with the previous reports that many herbal extracts are not easily dissolved in water and other solvents (Domingues *et al*., 2013). Qualitative analysis showed the presence of various groups of active compounds. The results revealed the presence of carbohydrates, phenols, glycosides, volatile oils and saponins as the main compounds in *A. catechu,* however, alkaloids, flavonoids and tannins were also found in its fractions. This has been reported in the previous studies that alkaloids are the major bioactive compounds found in *A. catechu* in the form of arecoline, norarecoline, guvacoline, isoguvacine arecaidine, nicotine and homoarecoline (Peng *et al*., 2015). The acaricidal activity of alkaloids found in *A. catechu* has already been reported against *R. microplus* (Bigg and Purvis, 1976), however, the present study proved their efficacy against cypermethrin resistant *R. microplus*. The other useful bioactive compounds reported from *A. catechu* include fatty acids, cycloartenol, fernenol, ursonic acid, arborinol, arecatannins, procyanidins, epicatechin, catechin, jacareubin, liquiritigenin, chryoseriol, luteolin, quercetin and isorhamnetin (Jain *et al*., 2021). Similarly, many epidemiological studies have proved that uptake of flavonoids results in reduced risk of neurodegenerative diseases, osteoporosis, cancer, diabetes and coronary disorders (Pandey and Rizvi, 2009). The same mechanism of action can be the possible outcome of the acaricidal activity of flavonoids against cypermethrin resistant cattle tick. Other active group of compounds in *A. catechu* also have their role in the acaricidal activity against drug resistant ticks. It was further revealed in this study that same plant extract with different fractions had different active group of compounds in the qualitative analysis. This is not surprising because variations in climatic conditions, collection and cultivation of plants for extract production can cause variations in results (Heimerdinger *et al*., 2006).

Such formulations are needed which enable rapid penetration into the ticks and protect the active ingredients from environmental degradation. Pharmacokinetic investigations are also necessary to ensure the standard use of plant extracts. It should be kept in mind that plant-based acaricidal products have an extremely promising potential for commercial market, especially when huge consumption of synthetic acaricides is considered. Along with the reduction in the development of resistance, these products will also be helpful for use in organic livestock farming (Adenubi *et al*., 2016; Štrbac *et al.,* 2021).

Conclusions: It was concluded from this study that crude extract of *A. catechu* and its petroleum ether and water fractions have a huge potential to be used as acaricide against cypermethrin-resistant *R. microplus*. This acaricidal activity was attributed to the presence of various active groups of compounds in the *A. catechu*. Petroleum ether fraction is a potential candidate for acaricidal drug development against resistant ticks.

Authors contribution: MUN performed the experiments and wrote the original manuscript. ZuDS designed and supervised the whole study. ZI gave valuable suggestions and revised the manuscript and BA helped while performing the phytochemical analysis.

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