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Evaluation of acaricidal activity of *Azadirachta indica* extracts against *Rhipicephalus* (*Boophilus*) *microplus* and its GC-MS analysis

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Abstract: Aim of present study was to evaluate the acaricidal activity of extracts of *Azadirachta indica*. The acaricidal activity of chloroform and hexane leaf extracts of *Azadirachta indica* and deltamethrin were tested on fresh larvae using larval packet test (LPT).

Azadirachta indica (neem) belonging to *Meliaceae* family is very important medicinal plant. The cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* is one of the most important ectoparasites of cattle. Traditional tick control is mainly based on the use of synthetic chemicals, However ticks are developing resistance to most of the available acaricides and also have many negative side effects. The LC_{50} and LC_{90} were highest for hexane leaf extract at 2139.34 and 8687.70 ppm, respectively. The LC_{50} and LC_{90} were lowest for deltametrin at 62.29 and 379.66 ppm, respectively. The chemical composition of extracts also analysed by modern sensitive gas chromatography–massspectrometry (GC–MS). In conclusion, phytogenic compounds having the acaricidal activity and also eco-friendly.

Keywords: *Azadirachta indica,* Acaricidal activity, *Rhipicephalus* (*Boophilus*) *microplus,* Larval packet test, Gas chromatography–massspectrometry.

Introduction

The cattle tick, *Rhipicephalus (Boophilus) microplus* is one of the most important ectoparasites of cattle and is widely distributed in tropical and subtropical regions including India (Ghosh *et al*., 2006). *Rhipicephalus (Boophilus) microplus* causes huge economic loss in cattle production by reducing weight gain, milk production and causing tick worry, blood loss, tick toxicosis and tick paralysis. Further, indirectly ticks involved in transmission of some bacterial, viral, protozal and Rickettsial diseases (Kirthi *et al*., 2011).

In an estimate, the control cost of ticks and tick borne diseases in dairy sector has been estimated to the $ 498.7 million per year and is responsible for economic losses that range over hundreds of millions of dollars per year (Guerrero et al., 2006). Keeping in view the

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impact of ticks and tick borne diseases on the individual and national economics the developing world should focus on tick control on priority basis (Bansal et al., 2005).

Present days synthetic chemicals are used to control the tick infestations. By its wider applications ticks are developing resistance to most of the acaricides (Ghosh *et al*., 2013). There is an urgent need to develop eco-friendly alternatives to chemicals. Alternatively, natural bioactive phytoacaricides are increasingly used for tick control as they have additional advantages such as low toxicity and more eco-friendly (Fernandes and Freitas, 2007). Natural products offer a cheap alternative to synthetic acaricides (Habeeb, 2010). Among the natural products, plant extracts and essential oils have been shown to have significant activity against economically important tick species (Ribeiro *et al*., 2011). One of the commonly cited advantages that may result from the use of botanicals for tick control is their biodegradability. Some plant extracts like i.e. *Azadirachta indica*, *Annona squamosa* (Magadum *et al*., 2008) *Ocimum basilicum, Spilanthes acmella, Polyalthia longifolia*, *Ficus benghalensis* and *Tephrosia vogelii* are capable of reducing tick feeding, moulting, fecundity and viability of eggs (Habeeb, 2010).

Azadirachta indica is commonly known as neem belongs to Meliaceae family, and is well known in India and its neighbouring countries for more than 200 years as one of the most versatile medicinal plant having a wide spectrum of biological activity. Every part of the tree has been used as a traditional medicine for household remedy against various human ailments, from antiquity (Avinash *et al.,* 2016). Kalakumar *et al*., (2000) and Srivastava *et al*., (2008) were also observed some acaricidal properties of its extracts. The aim of the present study was to evaluate the acaricidal activity of different extracts of *Azadirachta indica* to control *Rhipicephalus* (*Boophilus*) *microplus* and also characterize the chemical constituents in different crude extracts of the leaves of *Azadirachta indica* (neem) by using modern sensitive gas chromatography–mass spectrometry (GC–MS).

Materials and methods

Preparation of *Azadirachta indica* leaf extracts

The leaves of *Azadirachta indica* was collected from fields of different parts of Andhra Pradesh and identified by the Department of Botany, Sri Venkateswara University, Tirupati. The leaves were washed thrice in water to remove dust and dried under shade. Approximately 200 g of leaves samples were ground using a grinder machine. Finally the dry leaves samples were pulverized into powdered form. The leaf extracts were prepared by cold maceration as

described earlier by Sindhu *et al.* (2012a). Prepared extracts were refrigerated till further use at 4°C.

Evaluation of Acaricidal activity of leaf extracts against *Rhipicephalus* (*Boophilus) microplus*

Tick collection and larval bioassay

Engorged adult ticks were collected from heavily infested cattle in and around. The collected ticks were processed as per the standard procedure for morphological confirmation of *Rhipicephalus* (*Boophilus*) *microplus*. The processed ticks were examined microscopically and identified as *Rhipicephalus* (*Boophilus*) *microplus.*

The larval packet test was performed as per FAO (2004) to determine the *in-vitro* acaricidal activity of the test compounds. Engorged female ticks were obtained from the cattle in the study area, identified, cleaned, stored in a petri dish and maintained at 85-92% RH and 27.0 ± 1^{o}C. The female ticks were examined daily until oviposition. The eggs were separated and allowed to hatch in glass vials with cotton plug and kept in optimal conditions. The obtained seed ticks were maintained at 27.0 ± 1.0 ^{o}C and 85-92 % RH for 14-21 days. The larvae aged between 14 to 21 days were subjected to larval packet test. The dilutions for all the compounds were made in distilled water except for deltamethrin, which was diluted in olive oil with trichloroethylene (1:2).

Packets made of Whatman filter paper No. 1 (12 cm x 18 cm) were impregnated with 3mL of respective compounds and dried at room temperature for two hours. About 100 larvae were placed in acaricide impregnated filter paper packet and the top of the packet was sealed with a clamp (Plate 6). These larval packets were incubated at 27.0 ± 1.0 ^{o}C and 85-92 % RH for 24 hours. Mortality of larvae was assessed after 24 h of exposure. Lethal concentrations of the compounds were determined using the live and dead count. The percentage mortality in all of the experimental batches of larvae was corrected by applying Abbott's formula.

$$\text{Corrected percent mortality} = \frac{\%\text{ test mortality - }\%\text{control mortality}}{100\text{ - }\%\text{control mortality}} \times 100$$

Statistical Analysis

The mortality percentage from larval packet was subjected to probit analysis to calculate lethal concentration (LC_{50} and LC_{99}) for respective compounds using Statistical Package for Social Sciences (SPSS 19.0 V IBM, Illinois, Chicago). The lethal concentrations were expressed by 95% fiducial limits. The statistical difference between the lethal concentrations

were compared with the absence of overlap of 95 % fiducial limits. The level of significance was set at $p<0.05$.

Preparation of plant extracts for GC-MS analysis

Extraction procedure

Neem leaf powders (127 g) were extracted with methanol solvent (500 ml, 72 h) by using Soxhlet extractor. After extraction, it was filtered and the methanol solvent was evaporated completely by using rotary evaporator (Yamato Rotary Evaporator, Model RE 801). The solvent free methanol crude extract (9.6 g) was suspended in distilled water (100 ml). The suspension was transferred into a separatory funnel. Then it was extracted successively with different organic solvent with increasing polarity such as chloroform (1.00g) and hexane (1.327g), respectively (Hossain et al., 2011). All the crude extracts were filtered using filter paper to obtain particle-free crude extract. The defatted extraction procedure was repeated twice for all solvent for complete extraction and then filtered. The combined extracts were concentrated and evaporated by using rotary evaporator and dried under vacuum.

GC–MS analysis

This procedure was carried out with the slight modifications of (Hossain et al., 2011; Shah et al., 2010)The various crude extracts from the leaves of neem samples were analyzed using a Perkin Elmer GC–MS (Model Perkin Elmer Clarus 500, USA) equipped with a fused silica capillary column (30 m × 250 μm) coupled with a Perkin Elmer Clarus600C MS. An electron ionization system with ionization energy 70eV was used for the detection of compounds. Inert gas helium was used as a carrier gas at constant flow rate of 1 ml/min. Mass transfer line and injector temperatures were set at 240 and 260◦C, respectively. The oven temperature was programmed started from 50to 150◦C at 3◦C/min, then held for 10 min and finally raised to 300◦C at 10◦C/min. The crude samples were diluted with appropriate solvent (1/100, v/v) and filtered. The particle-free diluted crude extracts (1μl) were taken in a syringe and injected into injector with split mode. The split ratio was of 10:1. The percentage composition of the crude extract constituents was expressed as a percentage by peak area. The organic chemical compounds were identified and characterized in various crude extracts was based on GC retention time. The mass spectra were matched computer matching with those of standards available in existing computer library (VIT university, Vellore).

Results and discussion

Acaricidal activity against *Rhipicephalus* (*Boophilus) microplus*.

A modified version of FAO (2004) larval packet test (LPT) was used to determine the acaricidal activity against *Rhipicephalus (Boophilus) microplus* with various concentrations of deltamethrin; aqueous, hexane and chloroform leaf extracts of *Azadirachta indica*. The concentrations of deltamethrin varied from 25 to 400 ppm and the peak mortality (100%) were recorded at a concentration of 400 ppm. A total of eight concentrations of all the extracts viz., 437.5, 875, 1750, 2500, 3500, 5000, 6000 and 7000 ppm were used to test the activity. It was noted cent percent mortality was seen at 6000 ppm for aqueous leaf extract and 7000 ppm for both chloroform, hexane extracts respectively against *R (B). microplus* larvae. With the increase in concentration level the percent mortality rate also increased. The lethal concentrations of various compounds against fresh larvae (Table 1)

Table 1: Lethal concentrations of various compounds against fresh larvae

s.no	compound	LC_{50}	LC_{90}	SD	SE	R^2	Slope	Intercept
1	Deltamethrin	62.29 (38.24-101.46)	379.66 (233.07-618.43)	0.595	0.108	0.929	1.456	2.389
2	aqueous extract	1254.701 (750.10-1971.23)	5769.226 (3786.32-8790.56)	0.604	0.093	0.927	1.655	-0.250
3	Chloroform extract	1721. 65 (1133.79-2614.32)	8555.005 (NC-NC)	0.609	0.093	0.879	1.642	-0.362
4.	Hexane extract	2139.343 (1480.78-3090.78)	8687.70 (6013.34-NC)	0.529	0.082	0.942	1.889	-1.363

Values are inhibitory concentrations with 95% Fiducial Confidence Intervals in parenthesis
LC : Lethal concentration; SE = Standard error; SD = Standard deviation; NC= Not calculated
Probit analysis using IBM SPSS 19.0 V

Deltamethrin showed significantly ($P<0.05$) lower LC_{50} compared to respective plant extracts The LC_{99} values also in similar manner, aqueous extract showed least acaricidal activity with significantly ($P<0.05$) higher LC_{99} values.

Now a days deltamethrin is the most commonly used synthetic pyrethroid against the tick infestations in India. Due to its wide spread usage, *Rhipicephalus* (*Boophilus*) *microplus* are increasingly becoming resistant towards deltamethrin. In the present study hundred percent larval mortality was obtained with deltamethrin at a concentration of 400 ppm. Highest

resistance towards deltamethrin was reported from (Sharma *et al*., 2012) with less resistance (Jyothimol *et al*., 2014). But in the present study high resistance was observed for deltamethrin against *Rhipicephalus (Boophilus) microplus.* The reasons for the increased resistance might be due to its widespread usage, increase α/β esterase activity and target site insensitivity (mutation in para-sodium channel gene) (Kumar *et al*., 2013). To combat these resistance problems, the leaf extracts of *Azadirachta indica*, were found to be alternative. It was selected based on their wide pharmacological and medicinal uses and also by its previous acaricidal activity reports.

Al-Rajhy *et al.* (2003) detected the acaricidal activity of cardiac glycoside, digitoxin, cardenolide, azadirachtin and neem oil on *Hyalomma dromedarii*. The contact and dipping LC_{50} values of the extract and azadirachtin against tick larvae were 6.16, 20.3 µg and 587.7and 2500 mg lit^{-1} by performing the adult immersion test and the larval packet test, respectively. Srivastava *et al*. (2008) described the efficacy of 80 percent by the *Azadirachta indica* extracts against *Rhipicephalus (Boophilus) microplus* in India.

Magadum *et al*. (2009) evaluated the efficacy of *Azadirachta indica* and *Annona squamosa* extracts against *Rhipicephalus* (*Boophilus*) *microplus* in India. They could detect 71 percent of efficacy with *Annona squamosa* extracts against the *Rhipicephalus (Boophilus) microplus* by *in-vivo* but *in-vitro* methods showed the extracts from *Azadirachta indica* was more efficacious than the extracts of *Annona squamosa.*

Giglioti *et al*. (2011) detected the acaricidal activity of neem seed extracts against the *Rhipicephalus* (*Boophilus*) *microplus* in Brazil and showed the inhibiting egg laying and larval hatching rate by performing the adult immersion test. Zaman *et al.* (2012) evaluated the anti-tick efficacy of combined aqueous herbal extracts of *Azadirachta indica* leaves, *Nicotiana tabacum* leaves, *Calotropis procera* flowers and *Trachy spermumammi* seeds against the *Rhipicephalus* (*Boophilus*) *microplus* by using adult immersion test, larval packet test and ear bag method. They stated that the extract exhibited lethal effects on egg laying, hatching and total larval mortality.

Parte et al (2014) screened the acaricidal activity of aqueous extracts of *Azadirachta indica*, *Mangifera indica*, *Polyalthia longifolia, Annona squamosa* and Ficus benghalensis against the *Rhipicephalus (Boophilus) microplus.* They observed the combination of five plant extracts and showed 100 percent mortality as compared to a single plant extract. They concluded the effect of each plant extract required maximum time to get the 100 percent mortality.

Chemical composition of different extracts

The different organic crude extracts from the neem leaves were analyzed for respective compounds by GC–MS. Hexane crude extract after defatted of methanol crude extract of neem was analysed by using GC–MS had to be identification of 12 different groups organic compounds. According to their retention time on a fused silica capillary column. The chemical compounds were identified in hexane crude extract are listed in (Figs. 1 and 2and Table 2).

diphenylmethane, benzophenone, benzenemethanimineal pha.-phenyl, 4,8,12,16-tetramethylheptadecan-4-olide, tetratetracontane, octadecane, 3-ethyl-5-(2-ethylbutyl), nonacosane glyceryl monoricinoleate, dl-.alpha.-tocopherol alpha.-tocopherolquinone, stigmasterol and gamma.-sitosterol these are compounds of the hexane extract.

The defatted chloroform crude extract was analyzed by using GC–MS had leading to the identification of 3 different organic compounds using fused silica capillary column. The chemical constituents that were characterized in chloroform extract (Fig. 3) are Benzenemethanimine, Alpha.-phenyl-3-heptanol, 3,5-dimethyl and Phytol

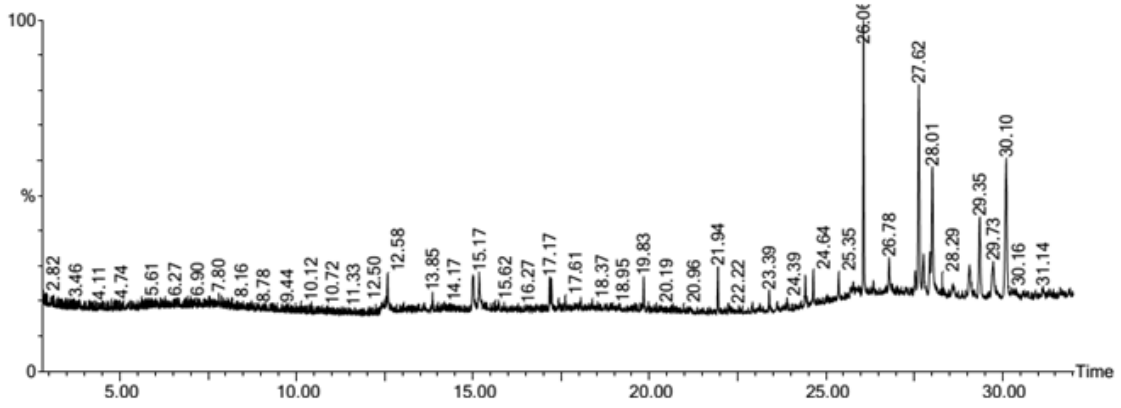
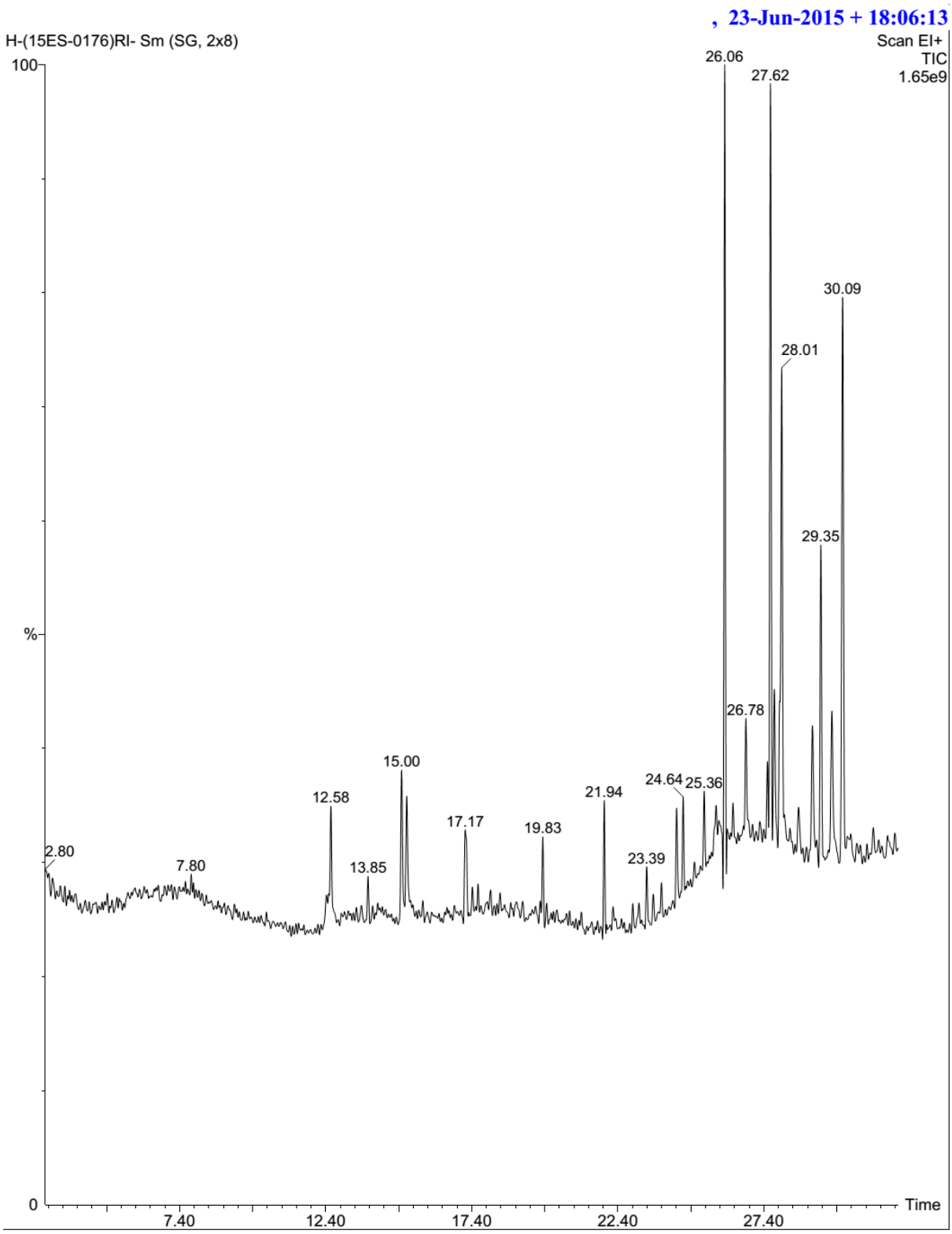


Fig: 1 Gas chromatogram of the chemical constituents of hexane crude extract from the leaves of neem

#	RT	Scan	Height	Area	Area %	Norm %
1	12.577	1955	228,116,880	7,556,735.0	2.441	12.54
2	14.998	2439	227,879,600	12,756,906.0	4.121	21.16
3	15.173	2474	233,039,232	9,564,534.0	3.090	15.87
4	21.936	3826	319,556,512	9,523,136.0	3.076	15.80
5	26.058	4650	1,851,745,024	54,358,920.0	17.560	90.18
6	26.783	4795	227,321,232	8,932,270.0	2.885	14.82
7	27.623	4963	1,386,946,688	60,276,800.0	19.472	100.00
8	27.758	4990	239,203,216	10,986,751.0	3.549	18.23
9	27.944	5027	267,696,272	10,535,233.0	3.403	17.48
10	28.009	5040	822,883,584	37,671,608.0	12.169	62.50
11	29.354	5309	549,325,248	29,473,594.0	9.521	48.90
12	30.099	5458	940,587,008	57,927,632.0	18.713	96.10



2: Gas chromatogram of the chemical constituents of hexane crude extract from the leaves of neem

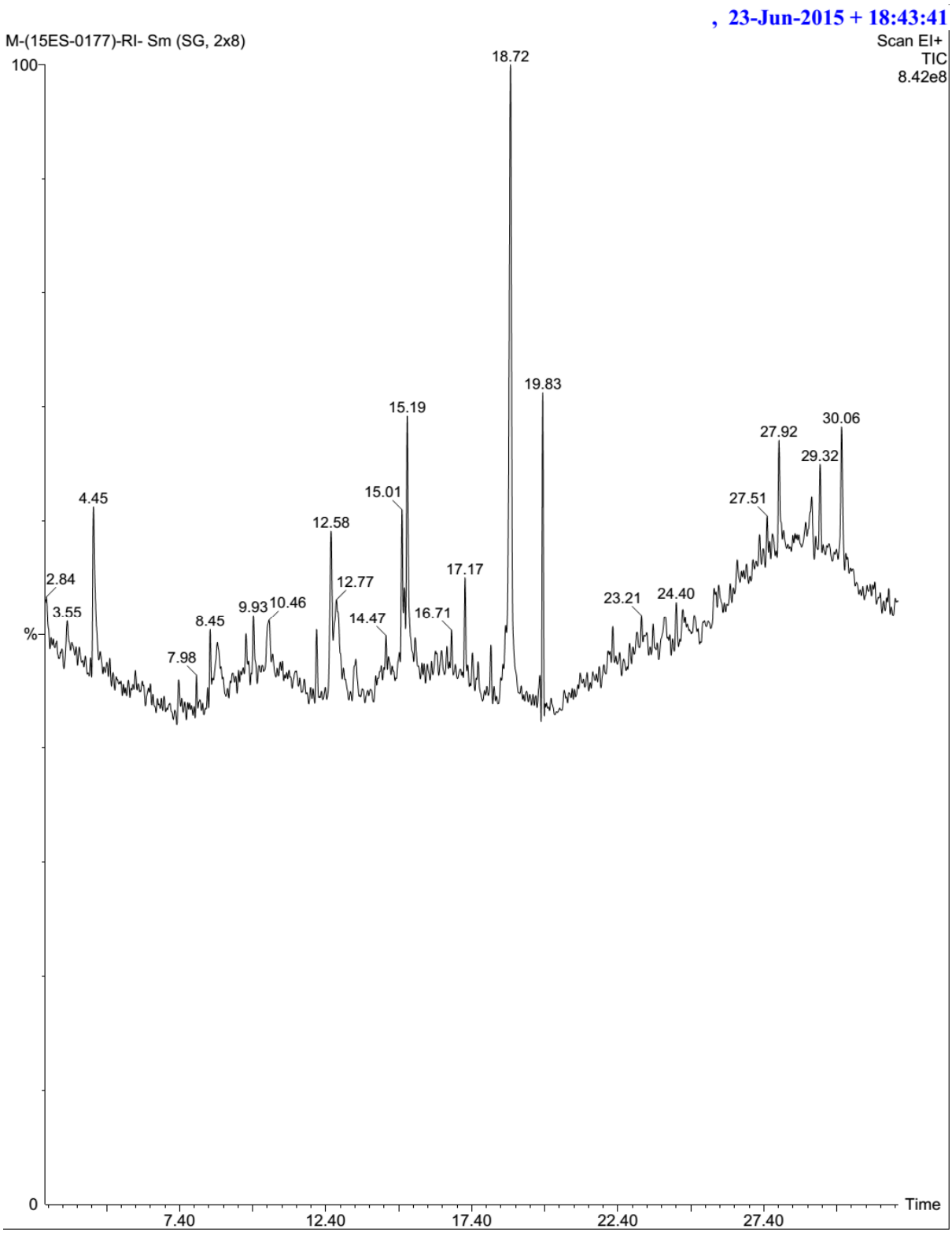


Fig: 3 Gas chromatogram of the chemical constituents of Chloroform crude extract from the leaves of neem

In this present study, aqueous neem leaf extracts showed highest acaricidal activity among the three crude extracts (Tab. 1). The identified compounds in different crude extracts from neem are hydrocarbon, terpenoids, phe-nolic, alkoloids and their derivatives.

Conclusion

Plant extracts have the have potent acaricidal activity and herbal eco-friendly nature. Phyto chemical analysis of neem revealed presence of hydrocarbon, terpenoids, phenolic, alkoloids and their derivatives that have been known for various biological effects. However, further studies are needed to studies on the toxicity, these extracts evaluated in-vivo for their acaricidal activity before their therapeutic application in veterinary practice. The present results of this study do not reveal that which chemical compound is responsible for different activity.

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