Plant infusions mediate oviposition of malaria, dengue and filariasis vectors: push-pull approach for vector surveillance and control

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ABSTRACT

Semiochemicals are small organic compounds that mediate interactions between organisms by transmitting chemical messages. They are used by insects for intra- and inter-species communication in their ecosystem. Mosquitoes transmit malaria, dengue, West Nile virus, chikungunya, yellow fever, filariasis and encephalitis to human beings across the globe. Mosquitoes perceive semiochemicals in their environment respectively emanating from potential hosts and habitats by the sensory apparatus and act wisely for the benefit of their progeny. Host seeking and/ avoidance, oviposition site seeking behaviors are governed predominantly by chemical stimuli along with visual cues that ensure blood feeding and subsequent egg laying to complete the gonotrophic cycle. Further, plant materials such as neem, tobacco and their infusions are naturally repulsive to mosquitoes while infusions of Bermuda grass could be a promising avenue for exploring natural attractant molecules using gas-chromatography coupled mass-spectrometry to identify biologically active molecules. In the laboratory studies one week old infusions of neem seed kernel and dried tobacco leaves reduced 90-97% egg deposition by gravid females of *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* mosquitoes, while oviposition substrates treated with one week old infusion of Bermuda grass, *Cynodan dactylon* received 40-60% increased egg laying compared to control. Through GC-MS analysis of these plant materials revealed several fatty acids, hydrocarbons, nicotine, thymol, ethyl dithioisoindole, δ-lactone, γ-elemene, squalene, eudesmenol and stigmasterol that could be responsible for the attraction and repulsion of mosquitoes to respective extracts. Being biological in origin, these semiochemicals are safe to humans and environment, when judiciously used would manage effectively the mosquito menace and mosquito borne diseases.

Keywords: Mosquito, Oviposition, *Cynodan* grass, NSKE, Tobacco, Infusions/extracts, GC-MS.

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INTRODUCTION

Oviposition activity of mosquitoes on a water body is a complex behaviour mediated by several factors involving physiological exigency, physical (visual, tactile) and chemical cues (Seenivasagan *et al*., 2009; Seenivasagan and Vijayaraghavan, 2010). Mosquitoes predominantly colonize in waters, wherein the senescent plant leaves get decomposed in water and provide good organic matter as food for growing larvae if gravid female had laid their eggs in such habitats. Visual cues from the oviposition site including colour and optical density of water, texture, temperature and moisture of the oviposition substrate, attract the gravid female from the distance and subsequently olfactory cues direct the female towards the specific oviposition site (McCall and Cameron 1995, Bandano and Regidor, 2002). Infusion in its crude form or the individual compounds present in infusion could be utilized in mosquito control programs by manipulating the attractiveness of existing oviposition sites (Allan *et al*., 2005).

Gravid females of *Aedes aegypti* ovipositing in organic infusions (Allan and Kline 1995; Ponnusamy *et al*., 2008; Ponnusamy *et al*., 2010) and egg deposition by *Culex quinquefasciatus* to infusions of Bermuda hay (Burkett–Cadena and Mullen 2008) in comparison to emergent aquatic vegetation, *Anopheles gambiae* avoiding Bermuda hay infusion baited traps (Eneh *et al*., 2016) demonstrate the efficacy of such eco-friendly approaches for mosquito control in respective ecological set-up. On the other hand, infusions from discarded cigarette buds mediating the oviposition behaviour of mosquitoes (Anderson and Davis 2014) and subsequent toxicity of cigarette butt waste to *A. aegypti* (Dieng *et al*., 2013, 2014) further emphasizes the applicability of such extracts/infusions in the field for mosquito control. The utility of neem wrt mosquito control is enormous and most of the published literatures describe the efficacy of neem oil as repellent (Mishra *et al*., 1995; Singh *et al*., 1996; Chandramohan *et al*., 2016) as well as its toxicity to immatures for different speicies of mosqutioes. However, Nagpal *et al*. (1995) showed wood scrapings in the shape of a ball and soaked in 5, 10 and 20% neem (*Azadirachta indica*) oil diluted in acetone, controlled *A. stephensi* and *A. aegypti* breeding in water storage overhead tanks (OHTs) with the application of these balls for 45-days.

Although, several studies have implicated certain volatiles emitting from the decay of plant materials such as 3-methylindole, 4-methylphenol, 4-ethylphenol, indole, and phenol (Allan and Kiline, 1995), and specific bacteria-associated carboxylic acids and methyl esters serve as potent oviposition stimulants for gravid *A. aegypti* (Ponnusamy *et al*., 2008), in India very limited work has been accomplished to utilize plant infusions for mosquito surveillance. Attraction of *Aedes albopictus* females to *Pennisetum* grass and paddy straw infusions (Gopalakrishnan *et al*., 2012), infusion from wood inhabiting fungus (Sivagnaname *et al*. 2001) influencing oviposition of mosquitoes has been studied in India. Therefore, in a renewed attempt to utilize these plant based semiochemicals, we prepared and evaluated the aqueous infusions of bermuda grass, neem seed kernel and dried tobacco leaves for mediating oviposition behaviour of three species of mosquitoes under laboratory conditions against *A. stephensi, A. aegypti and C. quinquefasciatus* which are vectors of malaria, dengue and filariasis in the Indian sub-continent. Further GC-MS analysis of these plant materials extracted with dichloromethane provides possible chemical signatures that may play a vital role in mediating oviposition responses of these vectors. We also report the push-pull strategy of vector mangement using these repulsive and attractive infusions in the laboratory which could be replicated in field conditions for eco-friendly mosquito control.

MATERIALS AND METHODS

Insects

The test mosquitoes of *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* were utilized from the cyclic mosquito colony maintained in our insectary under standard rearing conditions at 27±2°C and 70±10% RH for oviposition experiments. Adult mosquitoes were reared in wooden cages (75 × 65 × 65 cm) and 10% sugar solution ad libitum dispensed through a cotton wick in a Petri dish. A fowl for *Culex* and a rabbit for *Aedes* and *Anopheles* mosquitoes were used as a source of blood meal twice a week.

Preparation of Aqueous infusions

Neem (*Azadirachta indica* A. Juss) seeds and Bermuda grass, *Cynodan dactylon* (L.) were collected inside the DRDE campus, while dried tobacco (*Nicotiana spp.*) leaves were received from ICAR-CTRI, Rajahmundry. The grass was cut in to small pieces of 1-2 cm and shade dried. Infusion was prepared by adding 100 grams of these dried and chopped/crushed leaves of tobacco, Bermuda grass and crushed kernel of neem seeds in 1000 ml tap water in replicated flasks to get 10% solution. This infusion was kept in cotton plugged Erlenmeyer flask (3L) for 7 days at 27± 2°C temperature. Care was taken to ensure that the plant materials were immersed in water by intermittent swirling of flasks. Tap water (1 L) was also incubated in a separate flask for the same duration and used as control in all experiments. Other dilutions (5% and 1%) were prepared according to established procedures.

Oviposition Assay

Dual choice oviposition bioassay was carried out according to Seenivasagan *et al*. (2013) to observe the oviposition response of 5-7 day old 20-gravid females of *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* against 100 mL of 1, 5 and 10 % of respective infusions in 150 mL disposable cups. The duration of experiment was 24 hrs with four replicates for each concentration on a particular experimental day for a mosquito species. All oviposition bioassay were performed at 27± 2°C temperature and 65± 5% R.H. A negative control only with tap water was also conducted to observe the normal oviposition activity but not included for analysis. To study the push-pull effect choices were offered at 1% concentration of respective infusions to test mosquitoes in a separate experiment in which one cup contained either neem seed kernel infusion (NSKI)/tobacco leaf infusion (TBLI) against Bermuda grass infusion (BGI) in another cup. The number of eggs laid was observed after an elapse of 24 hrs. The number of eggs (egg rafts in case of *C. quinquefasciatus*) were counted manually and used for calculation of oviposition activity index which denotes the actual response of the insects to the test stimuli.

GC-MS analysis of extracts

All three plant materials weighing 1-gram each were soaked in 10 mL of HPLC grade dichloromethane overnight. The resulting extract was filtered and dried with sodium sulphate. The dried extract was used for GC-MS analysis. Precisely, 1 µl of this extract was injected into the injector port at 250°C for separation in a HP5-MS column (30m x 0.25mm x 0.25µ). Helium was the carrier gas with a flow of 1ml/min. The GC (Agilent 7890, MS 5975) was programmed at 50°C for 0 minutes with a ramp of 10°C/minute to 260°C and held for 5 minutes for a total duration of 26 minutes run. The mass spectrometer had the MS source 230°C and MS quadrupole temperature was set at 150°C. The eluting peaks were identified using NIST 2008 mass spectral library.

Statistical analysis

The mean oviposition activity index (Kramer and Mulla, 1979) values for the respective extract was calculated from four replicates obtained from the experiments. Two- way and 3-way ANOVA was performed on OAI values to observe any variation in the oviposition response profile between different species of mosquitoes / different plant infusions and concentration of the sources (Sigma stat. 2.03).

RESULT AND DISSCUSION

Oviposition response of mosquitoes

All the three species of mosquitoes showed positive oviposition response to Bermuda grass infusion at tested concentrations in a dose dependent manner. *Anopheles stephensi* laid 29%, 43%, 49% more eggs than control, while *A. aegypti* laid 27% eggs in 1% infusion and 54% more eggs in 5 and 10% infusions. The oviposition response to BGI by *C. quinquefasciatus* was not significant in 5 & 10% as 52-60% increased egg laying was observed than control. The aqueous infusion of crushed tobacco leaf elicited negative oviposition response from the test mosquitoes. All the test mosquitoes deposited significantly less number of eggs in treated substrates compared to control water. The range of negative oviposition at 1, 5, 10% infusions by *A. stephensi* was 65,72,75% to respective concentrations, while *A. aegypti* showed 61-67% reduced egg laying, whereas, *C. quinquefasciatus* showed 70-76% reduced oviposition. The differences among the concentrations were not significant for all the test species. To the NSKI both *A. stephensi* and *A. aegypti* showed dose dependent negative oviposition response respectively ranging from 66-80% and 44-63%, while *C. quinquefasciatus* showed 71-74% reduced oviposition in the treated substrates (Fig. 1). Significant statistical differences were observed among concentrations tested in the oviposition by all mosquitoes (Table 1).

Fig. 1. Oviposition response of *Anopheles stephensi, Aedes aegypti and Culex quinquefasciatus* to different concentrations (1%, 5%, 10%) of Bermuda grass infusion (BGI), neem seed kernel infusion (NSKI) and tobacco leaf infusion (TBLI) [Plant infusion, mosquito species and concentrations with different alphabet superscript based on OAI values are statistically significant P<0.001].

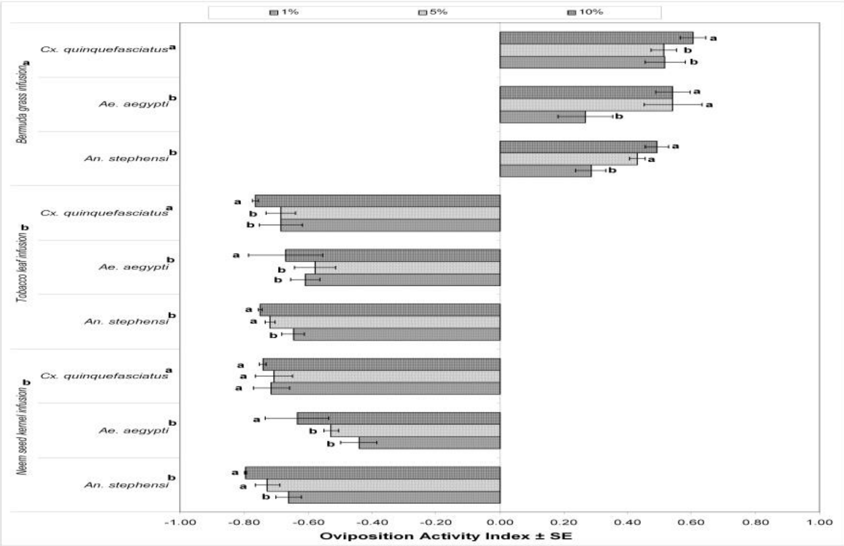


Table 1. Three way-ANOVA on the oviposition activity index (OAI) values of plant infusions against mosquito vectors

Source of Variation	DF	SS	MS	F	P value
Plant	2	1.003	0.501	38.753	<0.001
Mosquito	2	0.283	0.142	10.945	<0.001
Concentration	2	0.299	0.15	11.558	<0.001
Plant x mosquito	4	0.196	0.049	3.788	0.007
Plant x concentration	4	0.0586	0.0146	1.132	0.347
Mosquito x concentration	4	0.0546	0.0136	1.055	0.384
Plant x mosquito x concentration	8	0.0615	0.00769	0.594	0.780
Residual	81	1.048	0.0129		
Total	107	3.004	0.0281		

Push-Pull strategy

All the three species of mosquitoes decisively avoided egg deposition in repulsive infusion and laid more eggs in BGI treated oviposition cups. Both NSKI and TBLI at 1% pushed the females to BGI for oviposition demonstrating the pushing effect of respective infusions. In contrast the OAI of Control vs BGI was (+0.34) compared to push-pull experiment (+0.69). Oviposition activity indices subjected to 2- way ANOVA revealed significant differences in push-pull challenge, wherein Control Vs BGI received 50% lesser eggs compared to NSKI and TBLI vs BGI. The oviposition response and their interaction was not significant (Table 2, Fig.2) among the push-pull setup infusions/mosquito species.

GC-MS identification of volatiles

GC-MS analysis of tested plant extracts revealed both qualitative and quantitative variation in the identified chemicals (Table 3-5, Figs.3-5). Tobacco extract contained 23 chemicals followed by *C. dactylon* extract with 17 chemicals and neem seed kernel with 14 volatiles that are distinct from each other. However, few volatiles were common in all three extracts were also detected. Tetradecanoic acid, tetramethyl hexadecenol, hexadecanoic acid and octadecanoic acid were identified in all three plant extracts.

Figure 2. Oviposition response of *Anopheles stephensi, Aedes aegypti and Culex quinquefasciatus* females to Bermuda grass infusion (1%) in a push-pull set-up

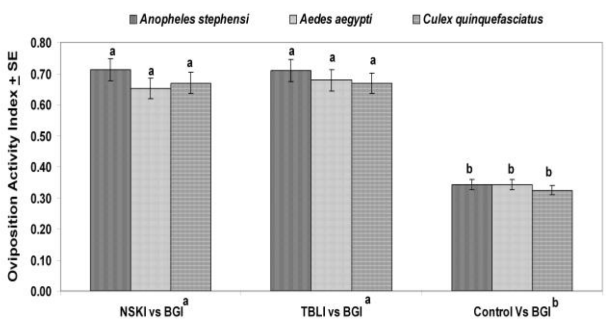


Table 2. Two way-ANOVA on the oviposition activity index (OAI) values of push-pull challenge (NSKI vs BGI, TBLI vs BGI, Control vs BGI) against mosquito vectors

Source of Variation	DF	SS	MS	F	P value
Insect	2	0.0030	0.0015	0.139	0.871
Challenge	2	1.0270	0.5140	47.419	<0.001
Insect x Challenge	4	0.0118	0.00295	0.273	0.893
Residual	27	0.2930	0.0108		
Total	35	1.3350	0.0381		

Heptadecane was noticed in NSK and *Cynodan* extracts, while phytol and octadecatrienoic acid was observed in tobacco and *Cynodan* extracts. Based on the respective peak area of chemicals to the total peak area of identified peaks after the integration of total ion chromatogram, it was observed that nicotine constituted 66%, hexadecanoic acid- 6% and octadecatrienoic acid- 4.5% of total compounds identified from tobacco leaf extract. In the NSK extract 43% was cis-13-octadecenoic acid followed by 18% of hexadecanoic acid, glyceryl acetate- 8.2%, stearic acid- 6% and 2-decenal 4.2% were present in major proportion among other volatiles. In case of *Cynodan* grass extract hexadecanoic acid- 30%, octadecatrienoic acid- 22%, and ocadecadienoic acid- 18% and 5% each of octadecanoic acid and tetramethyl hexadecenol constituted among total identified volatiles.

DISCUSSION

In the present study we examined the oviposition response of *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* to infusions of three plant materials viz, Bermuda grass, neem seed kernel and dried tobacco leaf in different dilutions ranging from 1, 5 and 10%. Gravid females deposited increased number eggs onto Bermuda grass infusion while neem seed kernel and dried tobacco leaf infusion caused 90-95% reduction in egg deposition by gravid females. It has been reported that plant materials, near the water resources get fermented and released various types of volatile chemicals which influence the female gravid mosquitoes for oviposition (Kramer and Mulla, 1979). The results suggest that the infusions released some volatile chemicals which act as chemical cues for gravid mosquito and help in selection of oviposition sites. The metabolic products formed by the microbial decomposition of organic matter present in water, attract the gravid mosquitoes, by communicating to female with reference to suitability of site and food availability for its next progeny (Santana *et al*., 2006; Ponnusamy *et al*., 2010). A number of studies have evaluated and identified such cues originating from a wide range of plant infusions including oak leaves, Bermuda grasses (Obenauer *et al*., 2009), infusion from wood inhabiting fungus (Sivagnanme *et al*., 2001) and a microencapsulated pheromone with aged infusions of *Oxalis per-carpae* (Michaelakis *et al*., 2009) influencing the oviposition by target mosquitoes in respective experimental set-up. Also the aquatic macrophyte volatiles influencing the oviposition behavior of *C. quinquefasciatus*, *A. aegypti* and *A. quadrimaculatus* females (Turnipseed *et al*., 2018) have been reported.

In this study neem seed kernel and tobacco leaf infusion exhibited dose dependent negative oviposition response ranging from 90-95%. Infusions of discarded cigarette buds (Anderson and Davis, 2014) reduced the oviposition by *A. Aegypti* and was also found toxic to *A. aegypti* (Dieng *et al*., 2013, 2014), which further emphasizes the field applicability of tobacco leaf infusion for mosquito control. Neem has been utilized for mosquito control wherein, the neem oil was tested as repellent (Mishra *et al*., 1995; Singh *et al*., 1996; Chandramohan e*t al*., 2016) as well as for its toxicity to immatures against different speicies of mosqutioes. Application of ball shaped wood scrapings soaked in 5, 10 and 20% neem oil diluted in acetone, controlled *A. stephensi* and *A. aegypti* breeding in water storage overhead tanks for 45 days (Nagpal *et al*., 1995). In addition to reduced egg laying in the NSKI treated oviposition substrates, death of mosquitoes (20-30%) with retained eggs was also observed in all species of mosquitoes at the end of 24 hr experimental duration. Gravid females dying with retained eggs while encountering highly unsuitable oviposition sites has been documented (Seenivasagan and Guha 2015; Seenivasagan *et al.*, 2015) for synthetic repellents. Plant extracts contain various chemicals, particularly hydrocarbons and fatty acids that are directly and indirectly involved in the oviposition process of mosquitoes and have been reported to play a major role (Barbosa *et al*. 2010; Hwang *et al*. 1984; Kominkova *et al*., 2012; Sivakumar *et al*., 2011). We observed tetradecanoic acid, tetramethyl hexadecenol, hexadecanoic acid and octadecanoic acid in all three plant extracts, while phytol and octadecatrienoic acid were observed in tobacco and *Cynodan* extracts.

Table 3 Compounds identified in Bermuda grass extract.

RT (min)	Name of the compound
12.83	Benzofuranone
12.98	Dodecanoic acid
13.46	6,9-dimethyl tetradecane
14.59	Heptadecane
15.23	Tetradecanoic acid
15.49	Trimethyl oxabicyclo heptyl propenyl acetate
15.67	Octadecane
16.10	Tetramethyl hexadecenol
16.17	Hexahydrofarnesyl acetate
17.37	Hexadecanoic acid
18.63	Trimethyl tetradecane
18.78	Phytol
19.02	Octadeca dienoic acid
19.08	Octadeca trienoic acid
19.22	Octadecanoic acid
19.53	Heptacosane
20.92	δ-Lactone

Several fatty acids identified from conspecific eggs have been reported to attract and repel *A. aegypti* (Ganesan *et al*., 2006) and fatty acid esters have been shown to influence oviposition and orientation response of *A. aegypti, A. albopictus, A. stephensi and C. quinquefasciatus* (Seenivasagan *et al*., 2013; Sharma *et al*., 2008; Sharma *et al*., 2009) under laboratory conditions. Heptadecane was noticed in NSK and *Cynodan* extracts and hydrocarbons influencing the oviposition response of *Aedes* mosquitoes are evident (Gonzalez *et al*., 2014; Seenivasagan *et al*., 2009) as many other hydrocarbons such as pentadecane, hexadecane, octadecane, heptacosane and octacosane have been identified in these test plants.

Table 4. Compounds identified in neem seed kernel extract

RT (min)	Name of the compound
9.175	2-Decenal
11.563	γ-Elemene
12.263	Pentadecane
13.457	Hexadecane
14.592	Heptadecane
15.222	Tetradecanoic acid
16.098	Tetramethyl hexadecenol
17.321	Hexadecanoic acid
19.027	Cis-13-octadecenoic acid
19.198	Octadecanoic acid
20.274	Heptacosane
20.592	Octadecenyl pyrrolidionone
20.915	Methyl tetradecenol acetate
24.468	Glyceryl acetate

The infusions/extracts could be used alone or in combination to demonstrate the push-pull strategy of integrated pest management. Manipulating the oviposition behavior of mosquito is a useful tool in determining the preference for oviposition sites by the gravid females and further application of attractant with combination of some lethal substance for control of peri-domestic container breeding mosquitoes. The present study demonstrates the potential of these plant infusions for deterring/stimulating egg deposition by target mosquitoes in oviposition traps.

Table 5. Compounds identified in tobacco leaf extract

RT (min)	Name of the compound
10.551	Nicotine
12.086	Nicotine oxide
12.175	Methyl quinolinamine
12.663	Trimethyl dodecadienylol
14.116	Oxo-∞-ionol
14.851	Cotinine
15.257	Tetradecanoic acid
15.663	Dodeca hydro boraphenalene
16.104	Tetramethyl hexadecenol
16.169	Trimethyl pentadecanone
16.992	Propyl nor-nicotine
17.38	Hexadecanoic acid
17.48	Indenone
17.745	Squalene
18.674	Eudesmenol
18.786	Phytol
18.957	Hepta triacontanol
19.074	Octadeca trienoic acid
19.227	Octadecanoic acid
20.092	Duva decatrine diol
20.927	Methyl tetradecenol acetate
24.527	Hepta cosane
24.844	Stigma sterol

Figure 3. Total ion chromatogram of Bermuda grass extract

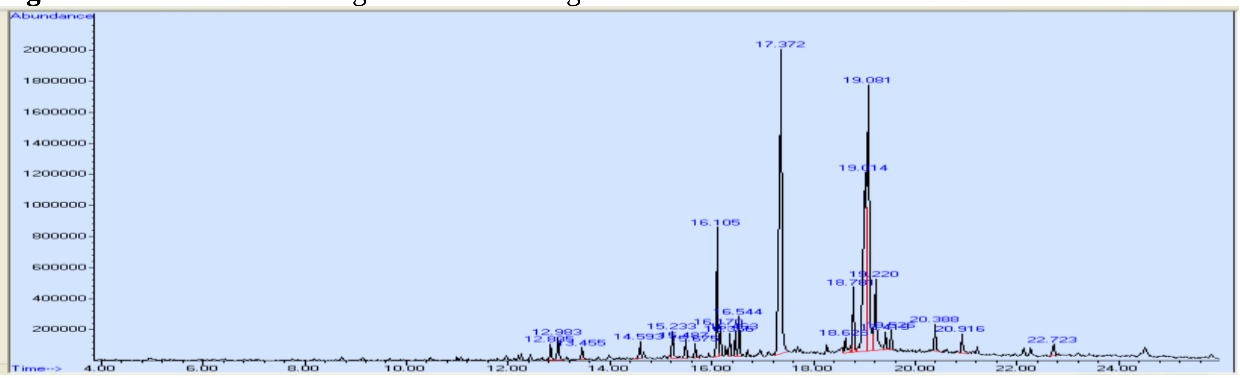


Figure 4. Total ion chromatogram of neem seed kernel extract

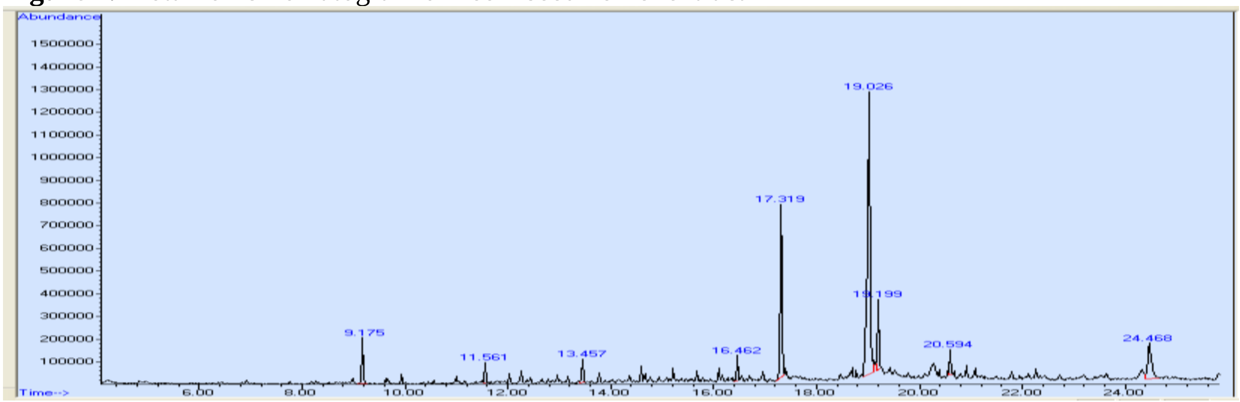
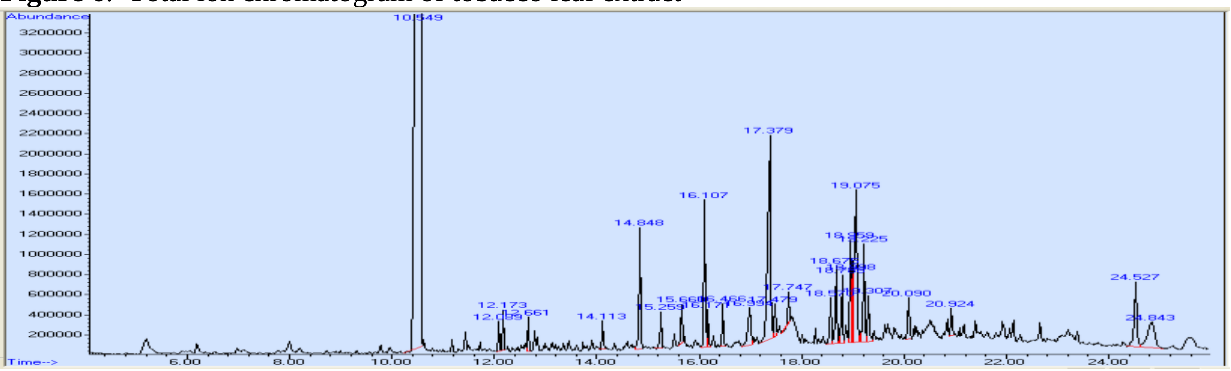


Figure 6. Total ion chromatogram of tobacco leaf extract



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