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Research article

ANTIMICROBIAL, MOSQUITO LARVICIDAL AND REPELLENT ACTIVITY OF ESSENTIAL OILS ISOLATED FROM THREE LOCAL SPECIES OF GINGER GROWN IN UPPER ASSAM REGION, INDIA

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Abstract

Background: *Synthetic mosquito repellents due to their side effects have led to the search for better, safer, and eco-friendly approaches. Essential oils are found to be a promising natural alternative with lesser or no harmful effects. Our study focuses on essential oils isolated from rhizomes of some local Zinger species collected from the upper Assam region of India.****Objective:*** *To extract essential oils from rhizomes of mango ginger, cassumunar ginger,and English ginger, to evaluate their mosquito larvicidal and repellent activities, and to study the antimicrobial susceptibility against 6 bacterial* strains viz. *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Bacillus subtilis*, and *Lactobacillus casei.* ***Materials andMethods:*** *In the present study extraction of essential oils was carried out in the Clevenger apparatus. The larvicidal activity was tested against larvae of Anopheles stephensimosquitoes. The dose-response study was carried out by the K and D module and the percentage of repellency was determined. Further, the antimicrobial activity was examined by the disk diffusion method, and the minimum inhibitory concentrations were determined. Lastly, the identification of essential oils wasperformed by GC-MS.* ***Results and Discussion:*** *On the basis of the study, English ginger (LC_{50} values 87.09 and 57.01mg/l at 24 h and 48h respectively) showed the highest larvicidal and repellency (80% at 0.21 mg/cm^2) potential amongst the three essential oils. The essential oils showed an inhibitory effect over the microbial strains. GC-MS study reported the presence of active natural*

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compounds in the essential oils. ***Conclusions:*** *Our study confirmed that the essential oils possess good larvicidal, repellant, and antimicrobial activity.*

Keywords: Antimicrobial; GC-MS; Mosquito larvicide; Repellent

Introduction

Vector Borne Diseases account for more than 17% of the estimated global burden of infectious diseases, causing more than 1 billion cases and over 1 million deaths annually[1]. Different methods have been developed for mosquito control including biological control, pesticide application, source reduction, sterile insect technique, physical exclusion (screens, nets, etc.), and discharge of genetically modified mosquitoes [2-3]. Biological control of the disease vectors is a better alternative to the chemical controls aimed against haematophagous mosquitoes. Mosquito repellents are extensively used in indoor and outdoor environments to avoid disease exposure [4-5]. However, repellents never guarantee complete protection, but could be minimize the chances of vector-borne diseases following proper applications [6]. They are useful for human activity coinciding with the enduring activity patterns of mosquitoes such as outdoor activities that take place at dusk and dawn, e.g., military operation in mosquito endemic areas or during hunting and fishing [7]. Mostly, mosquito repellent discovery has been possessed by the need to safeguard the military troops from vector human diseases(haematophagous arthropods). However, they have also been included in daily life of civilians owing increase incidences of mosquito-borne diseases [8]. Increasing resistant mosquito populations against synthetic repellents particularly pyrethroids and DEET (N,N-Dimethyl-meta-toluamide) has directed to explore newer alternative repellents. Further, toxicological and environmental concern of above instigated new investigations in the recent years is alarming. Better alternative to synthetic ones, the use of botanicals to control household insect-pest is being looked upon as a main source for safer and eco-friendly insecticide/pesticide. Since, botanicals are more eco-friendly, economic, species specific, biodegradable and have lesser or no harmful effects on non-target organisms including human being, they are preferable over synthetic ones[8-9]. At the same time it is necessary to control bacteria by synthetic or natural means [9].

The greatest use of essential oils (EOs) are for their therapeutic action, for flavoring, in perfumery or as starting materials for the synthesis of active compounds. For therapeutic purposes, they are administered as inhalations, orally, as gurgles, and mouthwashes and, some EOs are also utilized in the practice of aromatherapy.

Essential oils with high phenolic content show antiseptic properties, whereas others mostly act as carminatives [10]. The efficacy of EOs against different microbial strains depends on their chemical structure and various active components present in it. For instance, the antibacterial activity of thymol against *Staphylococcus aureus, Bacillus cereus,* and *Pseudomonas aeruginosa* to be comparable to carvacrol. However, in a research study, thymol and carvacrol were found to act separately against gram-positive and gram-negative bacteria [11]. The importance of the phenolic ring itself (destabilized electrons) is established by the lack of activity of menthol compared to carvacrol. In one study, the addition of an acetate moiety to the molecule appeared to increase the antibacterial activity; geranyl acetate was more active against a range of gram-positive and negative species than geraniol [12-13].

The northeast region (NER) of India is best known for its biodiversity, with wide variations in ecology, topography, and soil characteristics [13]. The climate of upper Assam is hot and very humid, with a high degree of moisture content all-round the year. The high humidity of this region pampers the growth of the microorganisms which grow and multiply rapidly due to the hot humid climate. The conducive climate of this region also favours cultivation of various ginger species including a very special ginger, known as Moran ginger. Literature survey does not provide sufficient information on the comperative insecticidal, repellancy and antimicrobial properties of different ginger species. Hence, the present study was designed to screen the effect of mosquito larvicidal, repellency, and antimicrobial activity of essential oils isolated from three species of locally collected rhizomes of ginger.

Materials and Methods

Collection of rhizomes

Fresh, healthy and disease free rhizomes of *Curcuma amada* (Mango ginger)*, Zingiber moran* (Cassumunar ginger) and Zingiber *officinale* (English ginger) were collected from MancottaTepor Gaon of Dibrugarh district, Assam, India, during the month of January 2016. All the specimens were identified by Dr. Pankaj Chetia, Assistant Professor, Department of Life Science, Dibrugarh University (Herberium No. DU/PHSc/HERB/03-05/2016). Essential oils used in this study, their biological source, local name and their part used are given in Table 1.

Table 1: Essential oils used in this study, their biological source, local name and their part used

Sl No.	Botanical name	Botanical parts		Local name	English name
		Whole plant	Rhizome		
1	*Curcuma amada*			Aamada	Mango ginger
2	*Zingiber moran*			Moran ada	Cassumunar ginger
3	*Zingiber officinale*			Jatiada	English ginger

Chemicals and reagents

Non-pathogenic microbial strains of *Staphylococcus aureus* (ATCC 9542)**,** *Listeria monocytogenes* (ATCC BAA-751)**,** *Bacillus cereus* (ATCC11778)**,** *Escherichia coli* (ATCC 25922)**,** *Bacillus subtilis*(ATCC 6051)**,** and *Lactobacillus casei* (ATCC 393) were collected from National Chemical Laboratory, Pune, India. Standard discs of Ciprofloxacin (5µg/disc)**,** ofloxacin (5µg/disc)**,** tetracycline (30µg/disc) and ampicillin (10µg/disc) were procured from Hi-media (Hi Media Laboratories Pvt. Ltd, Maharashtra, India).

Extraction of essential oils

The rhizomes were crushed in grinder and were set to air dry for 3 to 5 days. The materials were weighed to about 250 gm and subjected to hydro distillation by using Clavenger apparatus to obtain the essential oil at boiling temperature. The oil-water vapor mixture was condensed inside the cooling chamber of the apparatus and the oil gets separated. The obtained oils were collected in a sealed vial and kept at low temperature (4ºC) until further use.

Rearing of Anopheles stephensi

The test mosquitoes *Anopheles stephensi* were reared in laboratory conditions in wooden cages in the Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam, India. 10% w/v sugar solution was provided in cotton for nourishment and blood meal was provided by rabbit blood. A glass beaker containing 500 mL of water was kept for egg laying. The eggs were collected and transferred to a polypropylene tray containing three litres of water. Brewer's yeast powder provided as food supplement for the larvae. Collected pupae were kept in small cages (40 cm × 40 cm ×40 cm) for emerging into the adult. Female *Anopheles stephensi* (5-6 days old) were taken from the colony maintained at 27±2˚C and 70±5 % RH for repellent assay.

Larval bioassay

Third instar larvae of *Anopheles stephensi* was used for the experiments against EOs following the method described previously [14]. The EOs were tested in four different concentrations viz. 10 mg/L, 50 mg/L, 100 mg/L and 500 mg/L respectively in every glass beaker (100 mL) with 25 larva each. Acetone was used as a solvent and made the stock solution for each EO and added to those beakers to produce the desired concentration in 100 mL of tap water. After 24 and 48 h, a total number of dead larvae in each beaker were counted and corrected percentage of larval mortality was calculated.

Dose response study

Dose response study was carried out by the K and D module as per the method described by Islam et al. [15]. A volume of 0.025 mL of each concentration of the EOs in soybean oil viz. 0.02 mg/cm^2, 0.10 mg/cm^2 and 0.21 mg/cm^2 and 0.025 mL of the diluent (soybean oil) was applied randomly to the volunteer's marked skin as described previously [16]. After air drying for 5 minutes, the module was then placed over the marked area on the thigh and the sliding door was opened for the mosquitoes to access the treated areas. Each chamber of the K & D module contains 5 nulliparous, 5-7 days old mosquito (total 25 females per cage). The number of mosquito biting in each marked area was recorded for 1 min within every 5 min up to 25 min of repellent application [16]. Mosquitoes free from any exposure to repellent previously were used for each test. The effectiveness of EOs were determined by calculating the percentage repellency [17], using the formula described by Weaving and Sylvester in 1967.

$$\text{Repellency (\%)} = \frac{100 - \text{no. of bites on treated area}}{\text{no. of bites on control area}} \times 100$$

In vitro antimicrobial activity

Disk diffusion method

In vitro antimicrobial activity of the EOs of mangoginger, cassumunar ginger, and English ginger was evaluated as described elsewhere [18]. **S**tandardized bacterial strains of *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Bacillus subtilis*, *Lactobacillus casei* were used in this study. Empty sterilized paper disc (Whatman no.5, 6 mm in diameter) were impregnated with 50 µL of two different ratios (1:1 and 1:10) of the EOs under aseptic conditions, and placed on the agar surface with the help of sterile pointed forceps at a suitable distance apart so the respective disc can produce clear zones of inhibition around them (National Committee for Clinical Laboratory Standards, 1997). Sterile paper disc impregnated with aqueous DMSO was placed on the seeded petri plate as a vehicle control. Standard disc containing ciprofloxacin (5µg/disc), ofloxacin (5µg/disc), tetracycline (30µg/disc) and ampicillin (10µg/disc) of 6 mm diameter, was used as a reference control. Petri dishes were kept at 4 ºC for 1 h, and then incubated at 37 ºC for 24 h.The degree of sensitivity was evaluated in mm by measuring the visible clear zone of growth inhibition as a result of the diffusion of EOs from the respective discs into the surrounding medium on agar surface around the discs. The zones showing complete inhibition by the EOs compared with the standard drug containing a disc.

Minimum Inhibitory Concentration (MIC)

The EOs (10 mg) from all three ginger species were dissolved in 10 mL of 5% DMSO solution to obtain a concentration of 1000 µg/mL as a stock solution (0.5% v/v tween 80 was incorporated as a solubility enhancer). From the stock solution, 5 dilutions were prepared viz. 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, respectively. The inoculation of bacterial strains was prepared with both cultures and suspensions, and was adjusted to standard turbidity (10^5 CFU/mL). Then 1 mL of the stock solution was incorporated into 1 mL of Muller-Hinton broth to get different concentrations viz. 3.12 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, and 50 µg/mL, respectively. The standard suspension of the test organism (50µL) was transferred into each test tube. The control contained only the bacterial culture. Culture tubes were incubated at 37 ºC for 24 h and after incubation, MIC was evaluated [18].

Identification of components in essential oils

Essential oil samples were prepared and analysed as described by Hazarika et al. [14]. The EO samples were diluted in *n*-hexane at a concentration of 100 mg/L for evaluation under gas chromatography-mass spectroscopy (GC-MS), GC 7890B and

5977A MSD system equipped with a GC column J&W (HP-5 MS UI), (Agilent Technology, Germany). Helium was used as carrier gas at flow rate 1mL/min. Oven temperature was programmed from 40-300 ºC at 20 ºC/min. The injector temperature was set at 250 ºC and detector temperature was set at 230 ºC (quad) and 150 ºC (core), respectively. Components in the test samples were identified by NIST-14 mass spectra search software.

Results

Larvicidal activity

English ginger showed the highest larvicidal activity among the three EOs. It showed the LC_{50} (lethal concentration required to kill 50% of the population) values of 87.09 and 57.01 mg/L after 24 and 48 h, of post exposure respectively. The order of the EOs with their LC_{50} values (in mg/L) after 24 h of exposure were, English ginger (87.09)>cassumunar ginger (104.71)> mango ginger (176.19). After 48 h, the order was English ginger (57.01)>cassumunar ginger (86.16)> mango ginger (97.72). Fig 1 and Table 2 represents the larvicidal activity of EOs against the larvae of *Anopheles stephensi*.

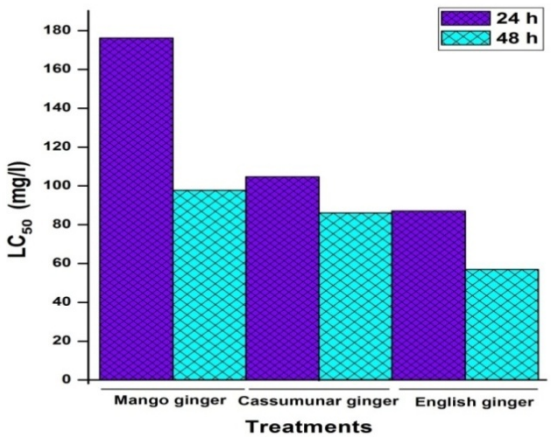


Fig 1: Larvicidal activity of essential oils against *Anopheles stephensi*

Table 2: Larval bioassay of essential oils against the 3rd instar larvae of *Anopheles stephensi* mosquitoes

Essential oils	**LC_{50} (mg/L)**	
	24 h	**48 h**
Mango ginger	176.19	97.72
Cassumunar ginger	104.71	86.16
English Ginger	87.09	57.01

LC_{50}: lethal concentration required to kill 50% of the population

Dose response study

The essential oils showed good repellency activity against *Anopheles stephensi* mosquitoes. The English ginger oil showed 100% repellency at 100 mg/L up to 2 h, while the cassumunar ginger and mango ginger showed 87 and 83%, respectively. The standard repellent N, N Di ethyl benzamide showed 100% repellency for up to 6 h. The results are shown in Fig 2 and Table 3, respectively.

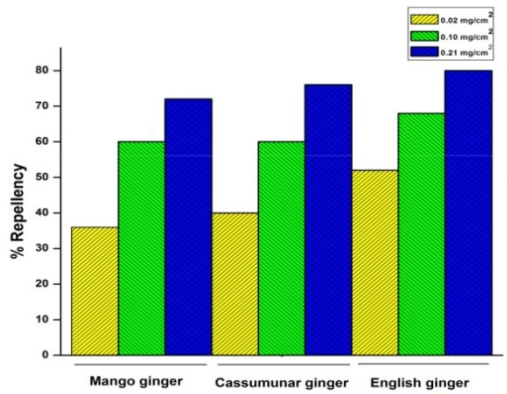


Fig 2: Dose response study of essential oils against *Anopheles stephensi*

Table 3:Dose response study of essential oils against *Anopheles stephensi* mosquitoes

Essential oils	Conc. 0.02 mg/cm^2		Conc. 0.10 mg/cm^2		Conc. 0.21 mg/cm^2		
	Mean No. of mosquito biting ± SD	% Repellency	Mean No. of mosquito biting ± SD	% Repellency	Mean No. of mosquito biting ± SD	% Repellency	ED_{50} (mg/cm^2)
Mango ginger	3.2±0.83	36	2.0±1.22	60	1.0±1.22	72	0.064
Cassumunar ginger	3.0±1.00	40	2.0±1.00	60	1.2±1.30	76	0.056
English ginger	2.4±1.14	52	1.6±1.14	68	1.0±1.00	80	0.050

Antimicrobial activity

Essential oils were found to be effective against the both gram positive as well as gram negative bacteria. Cassumunar ginger had shown the best results among the three essential oils. However, mango ginger showed a narrow zone of inhibition. The results have shown much impressive antimicrobial properties. The plates containing *Escherchia coli*, *Listeria monocytogenes and Staphylococcus aureus* showed 28, 27 and 40 mm of zone of inhibition for cassumunar ginger oil while the results for standard drugs (tetracycline) treated plated showed 34, 40, and 37 mm. The results for the antimicrobial activity of essential oils obtained from mango ginger, cassumunar ginger and English ginger, are shown in Table: 4-9 respectively.

Table 4: Zone of inhibition of mango ginger oil

Sl. no.	**Microorganism used**	**Zone of inhibition (mm)**	
		Test (Essential oil treated)	Standard (antibiotics treated)
1	*Escherchia coli*	10	27(Tetracycline)
2	*Listeria monocytogenes*	16	29(Tetracycline)
3	*Staphylococcus aureus*	21	31(Tetracycline)
4	*Bacillus subtilis*	12	27(Ciprofloxacine)
5	*Bacillus cereus*	11	16(Ampicillin)
6	*Lactobacillus casei*	13	22(Ofloxacine)

Table 5: Zone of inhibition of cassumunar ginger oil

Sl. no.	**Microorganism used**	**Zone of inhibition (mm)**	
		Test (essential oil treated)	Standard (antibiotics treated)
1	*Escherchia coli*	28	34 (tetracycline)
2	*Listeria monocytogenes*	27	40 (tetracycline)
3	*Staphylococcus aureus*	40	37 (tetracycline)

4	*Bacillus subtilis*	32	32 (ciprofloxacine)
5	*Bacillus cereus*	18	18 (ampicillin)
6	*Lactobacillus casei*	23	23 (ofloxacine)

Table 6: Zone of inhibition of English ginger oil

Sl. no.	**Microorganism used**	**Zone of inhibition (mm)**	
		Test (essential oil treated)	Standard (antibiotics treated)
1	*Escherchia coli*	14	28 (tetracycline)
2	*Listeria monocytogenes*	29	26 (tetracycline)
3	*Staphylococcus aureus*	26	32 (tetracycline)
4	*Bacillus subtilis*	27	32(ciprofloxacine)
5	*Bacillus cereus*	16	18 (ampicillin)
6	*Lactobacillus casei*	14	12 (ofloxacine)

Table 7:Minimum inhibitory concentration (MIC) of mango ginger

Bacterial strains	**Control**	**Concentration of the test oil (µl/ml)**				
		3.12	**6.25**	**12.5**	**25**	**50**
Bacillus subtilis	Growth	Growth	Growth	Growth	No Growth	No Growth
Listeria monocytogenes	Growth	Growth	Growth	Growth	No Growth	No Growth
Escherchia coli	Growth	Growth	Growth	Growth	Growth	No Growth

Table 8:Minimum inhibitory concentration (MIC) of Cassumunar ginger

Bacterial strains	Control	Concentration of the test oil (µl/ml)				
		3.12	6.25	12.5	25	50
Bacillus subtilis	Growth	Growth	No Growth	No Growth	No Growth	No Growth
Listeria monocytogenes	Growth	Growth	No Growth	No Growth	No Growth	No Growth
Escherchia coli	Growth	Growth	Growth	No Growth	No Growth	No Growth

Table 9: Minimum inhibitory concentration (MIC) ofEnglish ginger

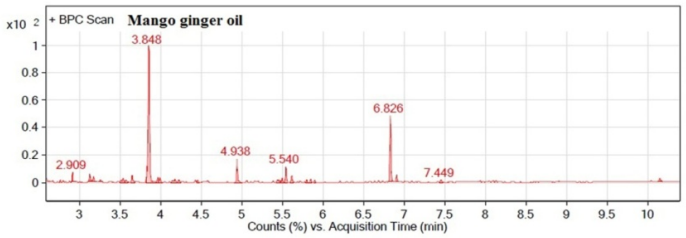
Bacterial strains	Control	Concentration of the test oil (µl/ml)				
		3.12	6.25	12.5	25	50
Bacillus subtilis	Growth	Growth	Growth	No Growth	No Growth	No Growth
Listeria monocytogenes	Growth	Growth	Growth	No Growth	No Growth	No Growth
Escherchia coli	Growth	Growth	Growth	Growth	No Growth	No Growth

Gas chromatographic-Mass spectrometric identification

From the GC-MS data, camphene, 1,8 epoxy-p-menthane, Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-,1S were found as the major aromatic compounds in mango ginger oil; Citral, thymol, succinic acid, carbonic acid, D-limonene, carveol, carene, and camphene in cassumunar ginger oil, and carene, linalool, endoborneol, alpha terpineol, citral, geraniol, thymol, neocloven in english ginger oil, respectively. The compounds identified by GC-MS are given in Fig 3.

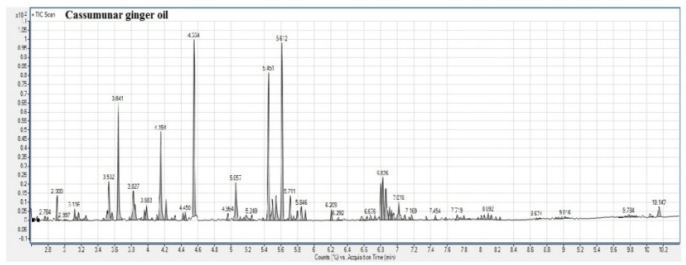
Discussion

Most ginger species grow naturally in damp, shaded parts of the low-land or on hilly slopes. They are easily identified by the characteristic aromatic leaves and fleshy rhizome when both of them are crushed and also by the elliptical to oblong leaves arranged in two ranks on the leaf-shoot. In Assam, members of Zingiberaceae family grow luxuriantly due to very conducive climate. The selected species were *Zingiber officinale, Curcuma amada and Zingiber moran* of which the former two are widely distributed across the world while *Zingiber moran* is endemic to the region (Assam). Herbs with antimicrobial, insecticidal and repellent properties have several advantages over the use of synthetic chemical agents. Natural products are easily accessible and the production cost is negligible as compared to the other synthetic ones. The development of insect resistance is very slow for plant products and they do not leave residues in environment.



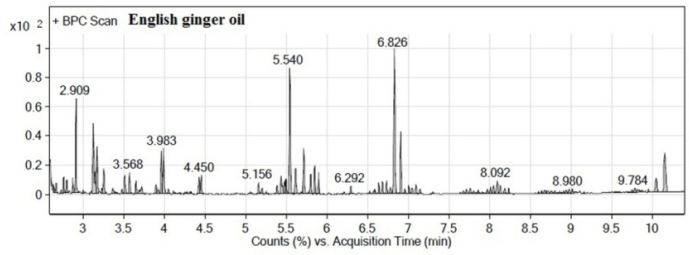
(a)

Sl no.	Chemical name	RT (min)
1	Camphene	3.646
2	1,8-Epoxy-p-menthane	4.17
3	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, 1S	4.938



(b)

Sl no	Chemical name	Retention time (mins)
1	3-Carene	4.216
2	Linalool	4.554
3	endo-Borneol	5.057
4	alpha.-Terpineol	5.182
5	Citral	5.451
6	Geraniol	5.493
7	Thymol	5.711
8	gamma.-Neoclovene	6.956



(c)

Sl no.	Chemical name	RT (min)
1	Citral	5.612
2	Thymol	5.711
3	Succinic acid	6.209
4	Carbonic acid	7.76
5	D-Limonene	5.405
6	Carveol	5.628
7	3-Carene	4.746
8	Camphene	3.843

Fig 3: GC-MS study of essential oils (a) mango ginger (b) cassumunar ginger (c) English ginger

Hence, EOs can be used to control mosquito population in human dwellings is an environmentally safe option as compared to synthetic larvicides and repellents. EOs from calamus, citronella, eucalyptus and thymus has been promising enough in killing mosquito larva [19]. A group of researchers found the LC_{50} of 50.78 mg/Lfor *Zingiber officinalis* Linn. against the larvae of *Culex quinquefasciatus*, the filarial vector [20].While another study suggested the LC_{50} values of *Zingiber officinale* as 40.5 mg/L against *Aedes aegypti* mosquitoes [21]. In our study the LC_{50} values of mango ginger, cassumunar ginger and English ginger were found to be 176.19, 104.71, and 87.09 mg/L against 3rd instar larvae of *Anopheles stephensi* mosquitoes, respectively. A study on repellency activity of *Zingiber officinale* against Culex reported 100% repellency up to 30 min at 2 mg/cm^2 concentration [20]. In our study we have three different doses applied as follows 0.02, 0.10 and 0.21 mg/cm^2. The ED_{50} for mango ginger, cassumunar ginger and English ginger were found to be 0.064, 0.056 and 0.050 mg/cm^2. Cassumunar ginger showed best repellent activity.

Our study reveals that, the essential oils from the rhizomes of mango ginger, cassumunar ginger and English ginger provides inhibitory effect over the microbial strains of *Escherchia coli, Listeria monocytogenes, Staphylococcus aureus, Bacillus subtilis, Bacillus cereus,* and *Lactobacillus casei*.The gram positive bacteria should be more susceptible than gram negative bacteria since they have only one outer peptidoglycan layer which is not effective permeability barrier. It has been reported that, the lipopolysaccharides present in the outer membrane of gram-negative bacteria gives the resistance towards antibacterial agents.

Conclusion

Essential oils were isolated from the field collected rhizomes of the selective species of Zingiberaceae family of upper Assam region and evaluated for mosquito larvicidal, repellent, antimicrobial activities. Identification of components of the essential oils was also carried out by GC-MS. Our study reveals that the EOs of *mango ginger, cassumunar ginger and English ginger* presented strong and broader spectrum of activity. The varying degrees of sensitivity of the bacterial strains may be due to both the intrinsic tolerance of microorganisms or the nature and combinations of different chemical components present in the EOs. As cassumunar ginger is an indigenous family found in the region, unrevealing its hidden potential would be a great achievement in new drug research. The oils showed good potential for larvicidal, repellent and antimicrobial activity. GC-MS results confirmed the presence of many important natural active components.

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