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Research Article

Dynamics of ethyl para-methoxycinnamate in *Kaempferia galanga* L. with rhizome age, storage and bioefficacy of rhizome extract against mosquito larvae

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Abstract
Kaempferia galanga is an important medicinal plant with a major active principle, ethyl para-methoxy cinnamate (EPMC), which has diverse medicinal applications. It has a great scope for cultivation to provide raw material for EPMC extraction. The present study assessed the EPMC Content and Yield/ha in the 1st year of cultivation, the loss of EPMC in stored rhizomes, and the mosquito larvicidal effect. The plants were harvested from the 3rd-9th month of planting; morphometric observations were recorded, and EPMC content (%) was estimated by the Thin Layer Chromatography-UV-Spectrophotometer method. The assessed ranges were for EPMC content (8.077% - 18.443%) and EPMC yield/ha (4.062 kg/ha - 16.977kg/ha) on oven dry weight basis. For storage study, 2.5 yr-old harvested rhizome was cut into small sizes (10g) and stored separately under different regimes (Light, Dark), conditions (Open, Closed), and durations (0, 30, 60, and 90 days). The EPMC content was evaluated after 90 days of rhizome storage. The maximum EPMC content (2.475%) was recorded in the materials stored in open-dark conditions, a 42.638% decrease from the initial storage. The Individual effect of storage condition revealed EPMC content was higher in material stored in the dark (2.747%) than in the light (2.679%), and higher in the open condition (2.882%) than in the closed condition (2.544%). The efficacy of the ethanolic extract of *K. galanga* rhizome on mosquito larval mortality was studied using 7 concentrations (0, 12.5, 25, 50, 100, 150, 200 ppm) applied for 4 hours. The LC_{50} value was found to be 66.433 ppm (LL: 32.234 ppm – UL:136.915 ppm) and LC_{90} value found to be 201.341 ppm (LL: 97.693 ppm – UL: 414.95 ppm).

Keywords: Ethyl para-methoxycinnamate, *Kaempferia galanga,* Mosquito larvae, LC_{50} , LC_{90} Thin layer chromatography, Storage.

INTRODUCTION

Kaempferia galanga L. is a perennial aromatic rhizomatous herb belonging to family Zingiberaceae . It is commonly known as Kenchur, Sand ginger, Aromatic ginger, Chandramul, and Sugandhabachha, and is distributed in the tropics and subtropics of Asia and Africa (Shirin *et al*., 2000). It is used as a medicine, a cosmetic, a spice, and a condiment. It is a source of valuable bioactive chemicals with good therapeutic effects on rheumatism, dry cough, colic, muscle discomfort, inflammation, and tumours, leading to its use as a folk medicine in China (Park *et al*., 2005; Liu *et al*., 2010; He *et al*., 2012). In the traditional practices of India, there also exists a rich history of utilizing this treatment for a variety of ailments, including respiratory issues like coughs and cold, headache, fever, skin disorders, pain-related disorders, arthritis, joint fractures, rheumatism, vertigo, wounds, gastritis and as an antidote for snake venom.

The biological activity of it has been scientifically proven as anti-inflammatory (Kumar, 2020 ; Ismiarni Koma-

la *et al*, 2018; Umar *et al*. 2012; Vittalrao *et al*. 2011 and Sulaiman *et al*. 2008) , treatment of intestinal wounds and urticarial (Nazar *et al*., 2008; Seth and Maurya, 2014), anticancer and cytotoxic to HeLa cells (Lallo *et al*., 2022; Ali *et al*., 2018 and Vincent K A, *et al*., 1992), anti-angiogenic effect (He *et al*. 2012), anti-tuberculosis activity (Lakshmanan *et al*., 2011), Anti-dengue (Kitani *et al*.,2018), hypolipidemic activity (Achuthan and Padikkala,1997), Hypopigmentary effect (Ko *et al*., 2014) and Cicatrizant (Parvez *et al*., 2005). According to sources, the volatile oil extracted from the plant's dried rhizome contains Pentadecane, Carvone, Eucalyptol, Methyl cinnamate, and Ethyl-p-Methoxy Cinnamate (Zhou *et al*., 2006; Zhang, 2007; Yang *et al*., 2018). EPMC is a major constituent of Kencur rhizome, which has anti-inflammatory, analgesic (Muhammad *et al*., 2012) and anti-angiogenic properties (Umar *et al*., 2014). The essential oil and other main compounds of this species are found to be effective larvicidal agents (Choochote *et al*., 2007; Liu *et al*., 2014) especially against *Aedes vittatus* and *Anopheles maculatus* without being harmful to aquatic life that is not the intended target (Mohamad *et al*., 2020). The phytochemical content in plant parts changes with the growth and development of the official part, with plant age, and also when harvested plant materials are stored for a period. This emphasizes the present study's aim to identify the optimal age for harvesting the rhizome to achieve maximum yield of this marker compound (EPMS), determine suitable storage conditions, and assess the effectiveness of the plant extract as a mosquito larvicide.

MATERIALS AND METHODS

K. galanga germplasm has been collected from Ekamravan medicinal garden , grown under Forest Department of Odisha, India under State Medicinal Plant Board. The planting materials raised in the Instructional farm of College of Forestry, OUAT from rhizomes, were transplanted at 1 month of age in the field with a spacing of 45cm x 45cm and suitable agro-techniques. The rhizomes of *K. galanga* L. were collected monthly from 3 months after planting through 9 months after planting. Each month is considered as a treatment and 3 replications were taken per treatment. The rhizomes were cleaned to be free of soil and other foreign materials, after which the fresh weight was recorded. The rhizomes were then dried in a hot-air oven at 47 °C and ground into a powder.

Procedure for extraction and separation of Ethyl para -methoxycinnamate (EPMC)

Oven dried powdered rhizomes of *K. galanga* L. was refluxed with ethanol (50ml) for 1.5 hours. The extract was cooled to room temperature, transferred to a beaker, and then filtered using a syringe filter. The filtrate was then made to a final volume of 50 ml, of which 0.05 ml was applied to a silica gel GF254 TLC plate using a pipette. The applied TLC plate was run with a solvent system containing n-hexane: ethyl acetate (9:1) to a distance of 12 cm for compound separation. The TLC plate developed with the solvent system was observed under a UV chamber, and a fluorescent blue spot was observed at an Rf of 0.45 (Fig. 3). The spot containing EPMC was then cut with scissors and dissolved in 5ml methanol. After complete dissolution of the compound, syringe filtration was performed, and the final volume was adjusted to 10 ml. Then, the spectral peak was observed under a UV-Vis spectrophotometer at 307.15 nm, which matches the reported peak absorbance at 307 nm of EPMC in Methanol (Suzana *et al*., 2011).

Preparation of standard curve of Ethyl para-methoxycinnamate (EPMC)

The methodology for standard curve preparation was standardized, and the ethanolic crude extract was applied to GF254 TLC plates and run with a solvent system of n-Hexane: Ethyl acetate: 9:1. The TLC plates developed by solvent system were observed under UV chamber. The spots containing EPMC from different plates were cut with a scissors and dissolved in 20 ml ethanol. After complete dissolution of the compound, syringe filtration was performed, and the ethanol was evaporated at room temperature. The dry weight of pure compound (12mg) was then recorded. The compound was dissolved in 50 mL of methanol to prepare a standard solution. From the standard solution, 1, 2, 3, 4, and 5 ml were pipetted into a test tube and diluted with methanol to a final volume of 5 ml. Then, these solutions were analysed by UV-Vis spectrophotometry at 307.15 nm. A graph was prepared (Fig. 2) from concentration value of compound against the absorbance value with a linear regression equation,y=21.607x-0.0034(R^2 = 0.98) having a Limit of Detection (LOD) for EPMC =0.00042 mg/ml and Limit of Quantification (LOQ) for EPMC=0.00127 mg/ml.

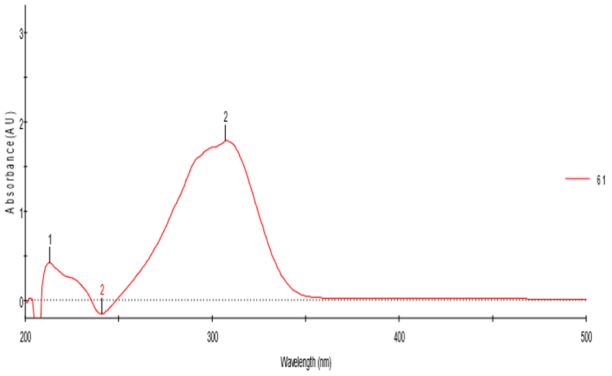

Fig.1. *Ethyl para-methoxycinnamate (EPMC) UV Spectral Peak*

Estimation of Ethyl para-methoxycinnamate (EPMC)

The absorbance of filtered pure compound extracted from a scrapped TLC plate was recorded at 307.15 nm, and the concentration of EPMC was calculated using the EPMC standard curveof EPMC . The percentage of EPMC was calculated using the formula below.

% age EPMC = [{(Y+0.0034)/21.607} x V_{EPMC} x (V_{ME}/ V_{TLC})] x 100 / W_{OD}

Where, V_{EPMC} : Volume (mL) of Pure EPMC Solution in Methanol

V_{ME} : Volume (mL) of Ethanolic extract of Rhizome

V_{TLC} : Volume (mL) of Ethanolic extract applied on TLC

W_{OD} : Oven dried weight of Rhizome

Y : Absorbance of Pure EPMC Solution in Methanol at 307.1nm

The data were statistically analyzed by RBD

Dynamics of Ethyl para-methoxycinnamate (EPMC) in different storage conditions

Material

The Rhizomes from 2.5-year-old *K. galanga* L. plant were collected from Instructional farm of College of Forestry, OUAT. These rhizomes were then oven dried. After drying, the dried rhizomes were cut into small tuber masses of 10 gm each.

Extraction, separation and estimation of ethyl para-methoxycinnamate (EPMC)

10 gm of tuber were stored in polybags and kept under light and dark conditions in closed and open containers for predetermined durations of 0, 30, and 60 days. The stored rhizomes were extracted, EPMC was separated by TLC, and EPMC was estimated under different storage conditions for each storage duration using the UV-Vis spectrophotometer method as mentioned earlier. The EPMC content was expressed in percentage on oven dry weight basis. The data obtained were statistically analysed using a factorial CRD.

Study the bio-efficacy of crude plant extract of *K. galanga* L. against mosquito larvae

Mosquito larvae were collected from stagnant water from natural source. Aedes and Culex larvae were identified in collected samples, from which *Aedes aegypti* 3rd instar (about 4mm in length) were taken for the experiment. The experiment was conducted in September, with room temperature of 28 °C and relative humidity of 82%.

The stock solution at 1000 ppm was prepared from a dried ethanol crude rhizome extract of 250 mg, initially mixed with 25 mL ethanol and finally diluted to 250 mL with distilled water. From the stock solution, 0 ml, 1.25ml, 2.5ml, 5ml, 10ml, 15ml and 20 ml of stock solution were taken separately in different beakers and added distilled water to make a final volume of 100 ml so that the solutions of 0 ppm, 12.5 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm respectively were prepared. The solvent prepared was allowed to stand at room temperature for 1 hour to evaporate some alcohol, and the loss was made up by adding water to a final volume of 100ml before transferring the larvae into the solutions. Each concentration represents Treatment and 3 replications/Treatment were considered.Then 10 no. of mosquito larvae collected from natural habitat were released to each replication in a beaker. These solutions were tested for its bio-efficacy against mosquito larvae. The mortality of mosquito larvae was recorded 4 hours after release. The LD_{50} and LD_{90} values of the extract were then estimated using probit analysis.

RESULTS AND DISCUSSION

Changes in Ethyl para-methoxycinnamate (EPMC) content and yield from rhizome with plant age

The variation in morphometric characters of *K. galanga* with growth and development of the plant found to be statistically significant in crown width and plant bio-

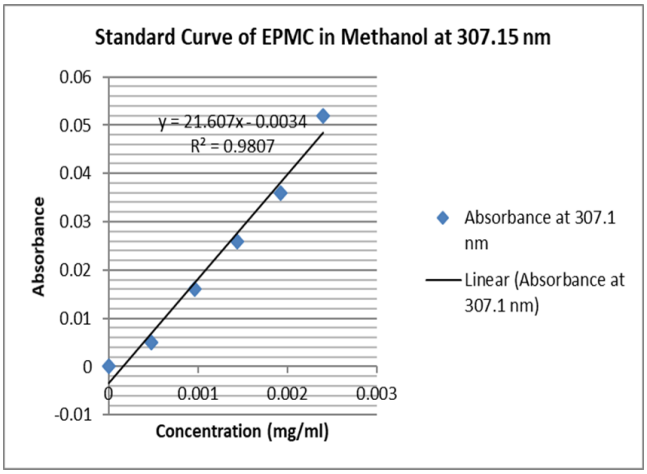

Fig. 2. *Standard curve of Ethyl paramethoxy cinnamate (EPMC)*

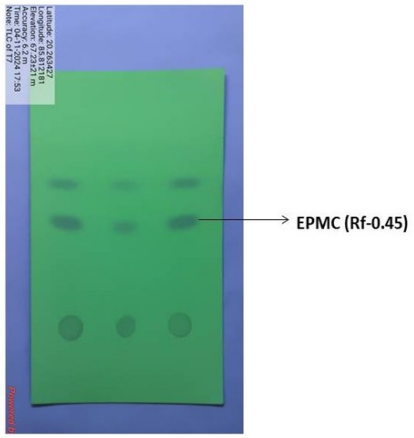

Fig.3. *TLC separation of Ethyl paramethoxy cinnamate (EPMC)*

Table 1. Changes in rhizome weight and ethyl para-methoxycinnamate (EPMC) content with plant age

Age of the plant	Fresh weight rhizome (g)	Oven dry weight rhizome (g)	Fresh wt. rhizome yield /ha (kg/ha)	Oven dry wt. rhizome yield /ha (kg/ha)	EPMC (%) on oven dry weight basis	EPMC Yield/Ha (kg/ha) on basis of oven dry weight
3 months	5.677	1.855	280.341	91.603	18.443 (25.336)	16.977
4 months	5.709	1.478	281.938	72.970	10.203 (18.553)	7.748
5 months	5.733	1.221	283.123	60.295	11.055 (19.273)	6.799
6 months	5.745	1.093	283.716	53.958	10.499 (18.557)	5.765
7 months	5.783	1.150	285.593	56.806	9.099 (17.459)	5.204
8 months	5.897	1.097	291.222	54.188	8.077 (16.426)	4.062
9 months	7.303	1.186	360.653	58.567	10.988 (19.285)	5.987
C.D.	N/A	N/A	N/A	N/A	5.040	6.255
F-Value	0.268	1.064	0.268	1.064	3.132	4.662
p-Value	0.94	0.435	0.941	0.435	0.044	0.011
SE(m)	1.136	0.270	56.091	13.334	1.618	2.008
SE(d)	1.606	0.382	79.324	18.857	2.288	2.839
C.V.	32.908	36.054	32.908	36.055	14.541	46.331

mass whereas statistically non-significant in all other characters like number of leaves, crown length (cm), fresh weight rhizome (g), oven dry weight rhizome (g), fresh weight rhizome yield/ha, oven dry weight rhizome yield/ha. The lack of significance in these characters is due to the plant's slow growth rate during the initial 9 months of the study. The rhizome from 3-month-old plants had significantly higher EPMC content (18.443%) and EPMC yield/Ha (16.977kg/ha) on an oven-dry weight basis (Table-1). This indicates a higher rate of biosynthesis of EPMC content (%) in early age, and, thereafter, the rate of biosynthesis decreases with increasing age of the rhizome, or EPMC is converted to other plant metabolites and transferred to other plant parts. A similar study has not been carried out for this species, but higher active principle content has been reported in younger plant parts than in mature ones in other species. Higher active principle content in initial stages reported in leaves of *Pelargonium* species, (Zakaria *et al*.,2015), leaves of *Thymus vulgaris* (Nezhadali *et al*., 2014), in leaves and stem of *Clinacanthus nutans* (Raya *et al*., 2014), plant and seeds of *Trigonella foeum* and *Foeniculum vulgare* (Singh *et al*., 2010), bud capitulum in *Spilanthes acmella* (Nayak and Chand., 2002).

Dynamics of ethyl para-methoxycinnamate (EPMC) content with storage

The plant parts harvested at an appropriate growth stage usually contain high levels of active principles, which must be properly stored under controlled conditions for further processing. As the processing units are far from the collection site, it is essential to dry and store the plant material until it is processed at the processing unit. Each active principle has a unique chemical structure, and its pharmacological and biochemical properties vary accordingly. The various degrees of stability of the active principle mainly attributes to its chemical structure. During drying, storage, and processing of the raw material, the active principle present in it decomposes and converts from one compound to another through enzymatic and microbial interactions in the presence of light, heat, air, etc. Enzymatic reactions occur at a faster rate in freshly harvested plant material in the presence of moisture at room temperature. However, the material also degrades even when the moisture content is reduced to a certain level due to its exposure to the external environment. To avoid this, harvested, dried material is stored in an appropriate container that serves as a barrier to the external environment and minimises degradation. To minimize degradation of phytochemicals, thermosensitive plants or active principles are stored at low temperatures, whereas light-sensitive materials are stored in the dark.

In case of stored Rhizome mass, the EPMC content during the interaction effect of different storage regimes, conditions, and durations was found to be statistically significant. For stored rhizome mass, EPMC content decreases from 0 days to 90 days of storage across all storage regimes and conditions. After 90 days of storage, the maximum EPMC content (2.475%) was recorded in the materials stored in open, dark conditions, with a decrease of 42.63% from the initial storage (Table-2 and Fig.4).

Table 2. Effect of interaction of storage regime, conditions and durations on ethyl para-methoxycinnamate (EPMC) content (%) in stored rhizome mass of *Kaempferia galanga*

Regime →	**Light**			**Dark**		
Condition →	**Open**	**Close**	**MEAN**	**Open**	**Close**	**MEAN**
0 Days	4.314 (2.305)	4.314 (2.305)	4.314 (2.305)	4.314 (2.305)	4.314 (2.305)	4.314 (2.305)
30 Days	3.074 (2.018)	1.788 (1.670)	2.431 (1.844)	2.935 (1.984)	2.222 (1.795)	2.578 (1.889)
60 Days	2.057 (1.748)	1.760 (1.661)	1.908 (1.705)	1.822 (1.680)	2.389 (1.841)	2.105 (1.760)
90 Days	2.070 (1.752)	2.055 (1.748)	2.062 (1.748)	2.475 (1.864)	1.510 (1.584)	1.992 (1.724)
Factors				C.D.	SE(d)	SE(m)
Regime X Condition X Duration				0.037	0.017	0.012

Table 3. Effect of interaction of storage regime and conditions on Ethyl para-methoxycinnamate (EPMC) Content (%) in stored rhizome mass of *Kaempferia galanga*

Condition → **Regime↓**	**Open**	**Close**	**MEAN**
Light	2.879 (1.956)	2.479 (1.846)	2.679 (1.901)
Dark	2.886 (1.958)	2.609 (1.881)	2.747 (1.920)
MEAN	2.882 (1.957)	2.544 (1.864)	
Factors	C.D.	SE(d)	SE(m)
Regime	0.013	0.006	0.004
Condition	0.013	0.006	0.004
Regime X condition	0.018	0.009	0.006

In the storage regime, irrespective of storage conditions and duration, the EPMC content in the stored rhizome mass was higher in material stored in the dark (2.747%) than in material stored in the light (2.679%) (Table-3 and Table-4). The maximum EPMC content in stored rhizome mass may be due to lower conversion of EPMC into other compounds, resulting from a comparatively lower surface area and darker conditions.

Irrespective of storage regime and storage duration, the EPMC content under different storage conditions was found to be higher in the rhizome tuber stored in open condition (2.882%) than rhizome stored in closed condition (2.544%) (Table-3 and Table-5). It may be due to a reduction in tuber moisture content after exposure to higher environmental temperatures, which promotes the conversion of other compounds into EPMC and prevents fungal or microbial decay.

Studies on bio efficacy of ethanolic rhizome extract of *K.galanga* L. against mosquito larvae

As vectors of numerous fatal illnesses, including dengue, malaria, filaria, yellow fever, and encephalitis, mosquitoes pose a serious global threat (Jang *et al.,* 2002). Resistance to chemical pesticides and changes

Table 4. Effect of interaction of storage regime and durations on ethyl para-methoxycinnamate (EPMC) Content (%) in stored rhizome mass of *Kaempferia galanga*

Duration→ Regime↓	**0 Days**	**30 Days**	**60 Days**	**90 Days**	**MEAN**
Light	4.314 (2.305)	2.431 (1.844)	1.909 (1.705)	2.062 (1.750)	2.679 (1.901)
Dark	4.314 (2.305)	2.578 (1.889)	2.105 (1.760)	1.992 (1.724)	2.747 (1.920)
MEAN	4.314 (2.305)	2.505 (1.867)	2.007 (1.733)	2.027 (1.737)	
Factors			C.D.	SE(d)	SE(m)
Duration			0.018	0.009	0.006
Regime X Duration			0.026	0.012	0.009

Table 5. Effect of interaction of Storage Conditions and Durations on Ethyl para-methoxycinnamate (EPMC) Content (%) in stored Rhizome mass of *Kaempferia galanga*

Duration→ / Condition ↓	0 Days	30 Days	60 Days	90 Days	MEAN
Open	4.314 (2.305)	3.004 (2.001)	1.939 (1.714)	2.272 (1.808)	2.882 (1.957)
Close	4.314 (2.305)	2.005 (1.732)	2.075 (1.751)	1.782 (1.666)	2.544 (1.864)
MEAN	4.314 (2.305)	2.505 (1.867)	2.007 (1.733)	2.027 (1.737)	
Factors			C.D.	SE(d)	SE(m)
Condition X duration			0.026	0.012	0.009

Table 6. Analysis of variance table for storage factors and their interactions

Source of variation	Sum of squares	Mean squares	F-calculated	Significance
Regime	0.003	0.003	9.350	0.008
Condition	0.070	0.070	231.764	< 0.001
Duration	1.755	0.585	1,943.213	< 0.001
Regime X condition	0.002	0.002	7.358	0.015
Regime X duration	0.009	0.003	9.712	0.001
Condition X duration	0.118	0.039	130.176	< 0.001
Regime X condition X duration	0.079	0.026	87.803	< 0.001

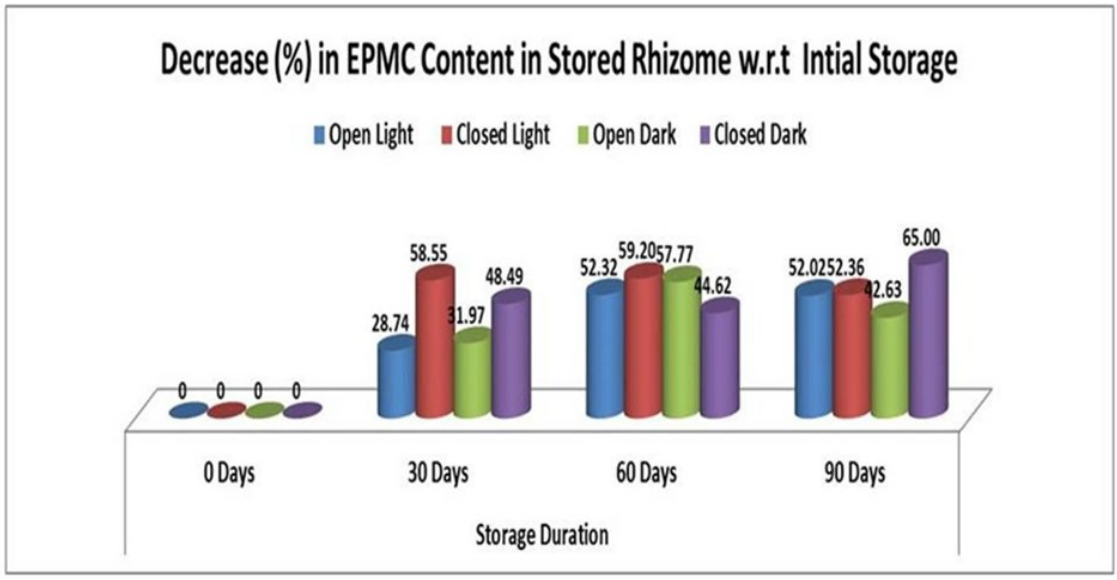

Fig. 4. *Decrease(%) in ethyl para-methoxycinnamate (EPMC) content in rhizome mass with respect to initial storage*

in morphological characteristics are indicators of their evolution. Both the ecology and non-target animals suffer from the use of chemical insecticides (Wattal *et al.,* 1981). Therefore, to limit the mosquito population, the right natural insecticide is required. Natural pesticides are safe to use and readily biodegradable (Redwane *et al*., 2002). Alsalhi *et al*. (2020) mentioned that *K. galanga* L. essential oil and its main compounds (EPMC: Ethyl p-methoxycinnamate, trans-ethyl cinnamate, and trans-cinnamaldehyde) are effective larvicidal agents against *Aedes vittatus* and *Anopheles maculatus* and have no toxicity to non-target aquatic fauna.

Bio-efficacy of ethanolic extract of *K. galanga* L. was carried out by taking 7 concentrations of the crude ethanolic extract on mosquito larvae as mentioned earlier. The mortality percentage of mosquito larvae after 4 hours of exposure was 90% at 200 ppm, 0% at the control, and 12.5% at 12.5 ppm. The LC_{50} value for *K. galanga* L. ethanolic extract dissolved in distil water was found to be 66.433 ppm with LL: 32.234 ppm – UL:136.915 ppm, and LC_{90} value found to be 201.341 ppm with LL: 97.693 ppm – UL: 414.95 ppm. (Table 7).

Table 7. Bio-efficacy test of ethanolic crude rhizome extract of *K. galanga* on mosquito larvae mortality (%) after 4 hours of exposure

Concentration of EPMC ethanolic extract (ppm)	No. of larvae tested	No. of larvae killed	Mortality (%)
200	10	9	90
150	10	8	80
100	10	8	80
50	10	2	20
25	10	2	20
12.5	10	0	0
0	10	0	0
LC50	66.433 ppm		
LC90	201.341 ppm		

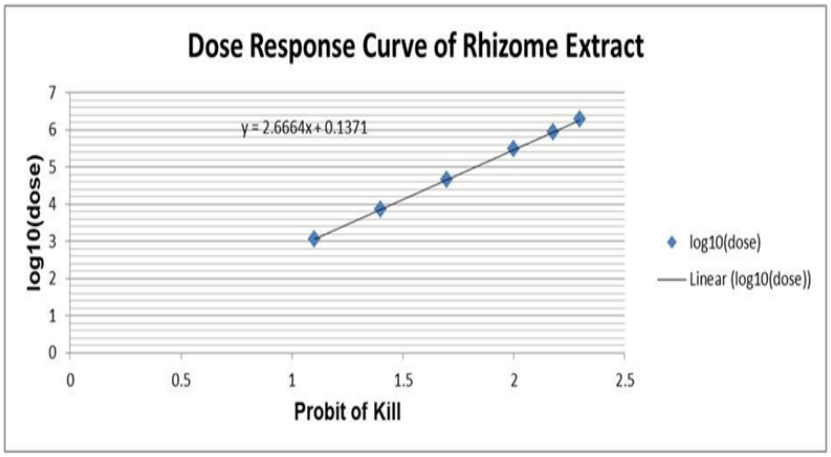

Fig. 5. *Dose response curve of Ethanolic rhizome extract dissolved in distilled water against mosquito larvae*

The ethanolic extract of the rhizome of *K. galanga* contains ethyl para-methoxycinnamate (EPMC), methyl cinnamate, carvone, eucalyptol, pentadecane, camphor, cineol, etc. Among all these, EPMC is the major compound. It has been reported that EPMC exhibits mosquito larvicidal activity (Ahn *et al*., 2008; Alsalhi *et al*., 2020). Besides EPMC, it has also been reported that 1,8-cineole (Thenmozhi *et al*., 2025), Carvone (Ganesan *et al*., 2023), and Camphor have 100% mortality of *Aedes aegypti* at 3% concentration and 50% mortality at 0.5% concentration (Shravani *et al*., 2023).The synergic effect of all these compounds may be responsible for killing the mosquito larvae. However, it cannot be exactly stated that which compound is responsible for mortality, so it needs to be tested separately. Similar tests were carried out by Choochote *et al*. (1999) using the hexane extract of the rhizome of *K. galanga*, which had an LC_{50} value of 42.33 ppm against mosquito larvae. Methanolic extract of the *K. galanga* rhizome reported to have a good larvicidal activity against *C. pipiens pallens, A. aegypti and O. togoi* (Yang *et al*., 2004) Pitasawat *et al*. (1998) also tested mosquito larvicidal activity in 10 plants out of which, *K. galanga* was also effective with LC_{50} value 50.54 ppm. The linear relationship between the probit of kill on the X-axis and log10(dose) on the Y-axis, given by the equation Y = 2.6664X + 0.1371 (Fig.5). The curve indicates that, with increasing dose, the response to kill mosquito larvae increases.

Conclusion

The above study concluded that for higher EPMC Content (%) and EPMC yield, the plant can be harvested after 3 months of age, as EPMC content decreases thereafter, while rhizome growth remains more or less the same. After harvesting, the dried Rhizome mass can be stored for 90 days in open polybags under dark conditions to minimise loss of EPMC content (42.638%) from the initial storage level. The crude ethanolic extract of *Kaempferia galanga* rhizome can be an effective natural larvicide for mosquitoes, with LC_{50} and LC_{90} values in a dissolved solution of 66.433 ppm and 201.341 ppm, respectively. The study on the larvicidal effect of EPMC can be conducted under natural conditions to assess the effects of the environment and the toxicity on other aquatic organisms. Further study is also required for a cultivation period of 2-3 years till a

constant yield of EPMC is achieved.

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Conflict of interest

The authors declare that they have no conflict of interest.

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