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Isolation, Characterization and Biological Activities of (2E, 4E, 6E)-11-Methyl Dodeca-2, 4, 6, 10-Tetraenoic Acid from Rhizomes of *Kaempferia Galanga*

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ABSTRACT- Plants contain various phytocompounds that shows different interactions with various proteins involved in several diseases. The ethanolic rhizome extract of *K. galanga* was shown a phytocompound (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid. Various biological activities like antioxidant, antimicrobial, protease inhibition and anti-proliferation activities were tested for (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid. The antioxidant activities with IC_{50} for (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid was shown 39.5 µg/ml and IC_{50} for standard (Ascorbic acid) was shown as 24.5µg/ml. The (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid were shown good antifungal activities (11 to 12 mm) along with antibacterial activities (11 to 13 mm). The bacterial standard like Tetracyclin shown zone of inhibitions as 10 to 14 mm and for fungi standard as Fluconazole shown zone of inhibition from 13 to 16 mm. The compound (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid were shown the good inhibition activity with enzymes like protease k and trypsin when compared with chymotrypsin. The standard tetracycline had shown the complete inhibition with proteolytic enzymes and control not shown the inhibition with proteolytic enzymes with X-ray photographic film. Dose Response of (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid from *K. galanga* on MCF-7 (Breast Cancer) cell line shown very less activity 80 µg/ml when compared with the standard tamoxifen 12.5 µgm/ml. Hence the experimentations concluded that a phytocompound, 1-(5-hydroxypentyloxy) propan-2-one from ethanolic rhizome extract of *K. galanga* shown good biological activities.

Key Words- (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid, *Kaempferia galanga,* Antioxidant activity, Antimicrobial activity, Protease inhibition and antiproliferative activities

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INTRODUCTION

Pharmacological properties of plants and their mechanisms have not been widely investigated in the perspective of present models in ageing diseases such as Alzheimer's, Parkinson's, Arteriosclerosis, Kidney stones, Cancer etc [1]. A phytocompound “gingkolides” isolated from *Gingko biloba* has shown neuroprotective, cholinergic and antioxidant activities appropriate to Alzheimer's disease mechanisms. The therapeutic efficiency is more and the side effects are minimal with extracts of Ginkgo n Alzheimer's disease

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compared to presently prescribed drugs such as donepezil or tacrine [2].

The earlier literature from medicinal herbs has a variety of medicinal plants like *Melissa officinalis* (balm) and *Salvia officinalis* (sage) that are showing memory-improving properties with a wide range of biological activities like astringent, anti-bacterial, fungistatic, virustatic, eupeptic, anti-hydrotic and cholinergic activities [3]. Hence the recent advances in understanding the ageing diseases has to re-explore the historical records for some new directions in drug development from the ancient knowledge on medicine [4].

The botanical assortment is required to early explorers and the later ethnobotany has played important roles in the expansion of new drugs in the past few centuries [5]. The four indigenous groups from Mexican Indians of medicinal plants are shown as Maya, Nahua, Zapotec and Mixe from ancient literature [6]. The sources indicate that the survival is a well-defined criterion showing specific for each culture. The various indigenous groups are used number of plant species for gastrointestinal illnesses from ancient times [7].

The people in developing countries are affected by new emerging microbial species like bacteria, viruses and protozoan's [8]. In various cases the drugs employed for treatment are useful, non-toxic and effective against resistance microbes. The abundant plant-derived natural products from unusual structural classes have been investigated as antimicrobial candidates including alkaloids, terpenoids, flavonoids and quinonoids [9]. *C. aromatica*, a closely associated species of *C. longa* contain curcumin, is of elevated caloric value and used as a replacement for baby food. Curcuminoids reveal antioxidant activity and free radical scavenging properties that perform as inhibitors of Human Immune deficiency Virus [10].

Curcumin (diferuloylmethane; 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3,5-dione), is a major bioactive secondary metabolite found in the rhizomes of turmeric (*Curcuma longa*) [11]. Turmeric is used as aromatic constituent for cooking in various parts of the world. Curcumin extracted from *C. longa* rhizome is an anti-inflammatory, anti-oxidant agent and has anti-carcinogenic properties [12].

Explore of new major plant sources should be a right way in current and future efforts toward sustainable maintenance and rational utilization of biodiversity [13]. The antioxidant activity in rhizomes of *C. aromatica* and *C. longa* in family member to curcumin and phenol quantity by using DPPH method has been reported in earlier studies [14]. Curcumin inhibits cAK, PKC and CDPK proteins in a fashion that are competitive with respect to both ATP and the synthetic peptide substrate [15].

The importance of medicinal plants and traditional systems in solving the healthcare problems of the world is gaining increasing interest [16]. There are growing amazingly at the international level repeatedly to the damage of natural habitats in the countries of origin. There is an adaptation of traditional medical practices and research in the developing countries that have an integral part of ancient and modern cultures [17]. Biotechnological approaches provide potential approaches in the production of food and medicinal supplements to traditional agriculture and industrial production of bioactive plant metabolites [18].

Traditionally, all medicinal preparations were derived from plants in the refined form of crude extracts as simple form of raw plant materials or mixtures [19]. Applications in various human cultures and societies recommend several thousands of plants that have been known to medicinal constituents in current generations [20]. The organized evaluation of indigenous pharmacopoeias using natural and synthetic drugs to contribute to the improved healthcare in marginalized regions has been placed on the agenda of international and national organizations [21].

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MATERIALS AND METHODS

Collection of plant materials

Fresh plants consisting of rhizomes from Zingiberaceae family, *Kaempferia galanga* were collected from Visakhapatnam District, Andhra Pradesh and some regions of Kerala during July and August 2011. The plant was authenticated by Dr. P. V. Arjun Rao, Head, Department of Botany, Phytopharma Technology Laboratory, and Visakhapatnam, India.

Extraction Process

Nearly 150 gms of air dried powder were taken with 800 ml of ethanol and plugged with cotton wool and then kept on orbital shaker for 48 hours with 150 rpm at room temperature. The extract was filtered with whatmann no 1 filter paper and collect the supernatant. Then solvent evaporated through rotavapour and make the final volume one-fourth of the original volume and stored at the 4ºC in air tight containers.

Isolation and Purification of (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid from Rhizomes of *Kaempferia galanga*

Chemicals which are used in this method as Silica gel G (Qualigens, India for TLC) Silica gel G (Qualigens, India for column chromatography). All the chemicals and reagents were used of analytical grade.

The column was packed using n-hexane. The filtrate of ethanol with 5.12 gms (w/w) was carried out to Silica gel G-60 (60-120 mesh). Then the column was first eluted with hexane followed by the polarity of the system raised by increasing order and ethyl acetate in ethanol (20:30)-(2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid was separated with fractions F19 to F32. 10ml fractions were collected and the fractions having similar compounds were pooled together after monitoring with thin layer chromatography. Visualization of the TLC chromatograms was achieved by using iodine vapors. The fractions were further subjected to phytochemical screening by using the TLC method; those fractions that gave similar spots and Rf with specific TLC methods were again pooled together into major fraction was (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid.

The compound purity was checked by using n-hexane and ethyl acetate as the solvent system on silica gel G TLC plates of 2 mm thickness. The iodine chamber was used to visualization of spots. The FT-IR spectra were recorded on perkin-Elmer spectrophotometer. The proton NMR spectra were scanned on a Bruker 400 M Hz. Spectrometer in methanol using TMS as standard and chemical shifts are expressed in δ ppm. The ESI mass spectra were recorded on an Agilent 6100 QQQ spectrometer (positive ion mode & negative ion mode).

Anti oxidant activity with DPPH

Standard: Ascorbic acid

Chemicals & Reagents

a. 100 µM of DPPH (1,1-diphenyl-2-picrylhydrazyl): About 3.9432 mg of DPPH is added in 3ml of methanol and the content obtained for 100 ml (final concentration 100 µM).
b. Stock solutions: About 3 mg/ml of stock solution is equipped in DMSO (or Dimethylsulfoxide).
c. Test solutions: Dilutions from 0 to 1000µg/ml of the test solutions were prepared.

The reaction mixture is prepared in addition of 20 µl of test solutions with 280 µl of DPPH reagent to obtain a final concentration of 300 µl. The solution is kept in incubation chamber in the dark for about 50 minutes and the absorvation was taken at 517 nm using the spectrophotometer. An IC_{50} value for anti-oxidant activity was confirmed as elicits that are about half maximal response.

Antimicrobial Activity

The antimicrobial activity was conducted based on zone method. Microbes from ATCC (American Type Culture Collection), USA have been used in the present study. Several bacteria used in the current research work are

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Bacillus subtilis (ATCC 6051) and *Klebsiella pneumonia (*ATCC 13883) that belongs to gr+ (gram positive) bacteria. *Serratia marcescens* (ATCC 14756), *Pseudomonas aeruginosa* (ATCC 15442), *Enterobacter aerogenes* (ATCC 13048) and *Escherichia coli* (ATCC 25922) belong to gr- (gram negative) bacteria. The fungi used in the present experimentation are *Aspergillus niger* (ATCC 6275) and *Candida albicans* (ATCC 10231). Antimicrobial activity has been conducted based on zone method.

Protein Inhibition Activity

Activity for protease inhibitor against proteases (Chymotrypsin, Protease K and Trypsin) was assayed in the present experimentation. In the present method, Trichloroacetic acid (TCA) soluble fractions that formed by action of Proteases with protein substrate Hammerstein casein were measured by change in the absorbance at 280 nm. The obtained residual caseinolytic activity for the trypsin in the presence of protease inhibitor at 37˚C was measured with inhibitory activity. Blanks for the substrate, inhibitor and enzyme were included in assay along with test.

Approximately 10 µl of protease inhibitor (plant extract) was mixed with 10 µl of protease (0.5 mg/ml) and was spotted onto a stripe of the X-ray film. 10 µl of protease was mixed with 10 µl of 0.lM (pH 7.0) phosphate buffer as the control and was spotted on to the X-ray film. The above inhibitor, protein and buffer mixtures were incubated of X-ray film at 37°C for 10 minutes. After 10 minutes, wash the film under tap water gently without touching other objects for the zone of gelatin hydrolysis. The protease activity will be visualized as thick color without gelatin hydrolysis and non-protease activity will be shown as zone of gelatin hydrolysis.

Antiproliferative Activity

As the ethanolic crude extracts has shown efficient antioxidant, antimicrobial and protease inhibition activities than other tested extracts, a preliminary investigation has been made for finding antiproliferative effects of ethanolic crude rhizome extract of *K. galanga* on MCF-7 (Breast Cancer) cell line.

Human cancer cell line (MCF-7) used in this study were produced from National Centre for Cell Science, Pune. The all cells were grown in Minimal Essential Medium (MEM, GIBCO) and addition with 2mM L-glutamine, 5 percent Fetal Bovine Serum (FBS) (growth medium) and 4.5 g/L glucose at 37˚ C in 5% CO_2 incubator.

The trypsinized cells were seeded in T-25 flask and in every well of 96-well plane-bottomed tissue culture plate at a concentration of $5x10^3$ cells/well in the growth medium were cultured at 37˚C in 5% CO_2 to adhere. After 48 hrs of incubation, the supernatant was redundant and the cells are pretreated with the growth medium. The cells were subsequently mixed with different concentrations of ethanol extracts of *K. galanga* (12.5, 25, 50, 100, 200 and 250 µg/ml) to reach the final volume of 100 µl and cultured for 48 hours. The test samples well separately prepared as 1.0 mg/ml concentrations of stock solutions using DMSO. The culture medium and the solvents are used as controls. Each well then added with 5 µl of new MTT (i.e 0.5 mg/ml in PBS) left for incubation about 2 hours at 37˚C. The supernatant with the growth medium was then discarded from the wells. The mixture is then replaced with 100µl of DMSO solution to solubilize the formazan product. After 30 min. of incubation, the absorbance or the OD of the culture plate was measured with ELISA reader (Anthos 2020 spectrophotometer model) at a wavelength of 492 nm.

RESULTS

The phytochemical investigation of the ethanolic rhizome extract of *Kaempferia galanga* shown good pharmacological activities due to containing a phytocompound at Rf value as 0.97 with TLC (Fig. 1)

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Fig. 1: TLC for Column elute of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

The data was established as (2E, 4E, 6E)-11-methyldodeca-2, 4, 6, 10-tetraenoic acid based on UV, Proton NMR, ^{13}C NMR, Mass and IR spectra's (Fig 2 to 8). Figure 9 shows the confirmed structure based on the observed spectra's.

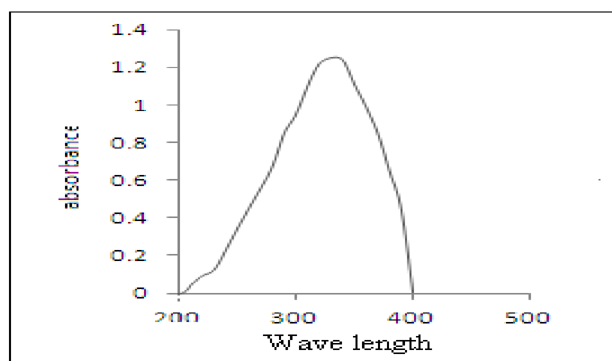

Fig. 2: UV Spectrum for ethanolic rhizome elute, (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

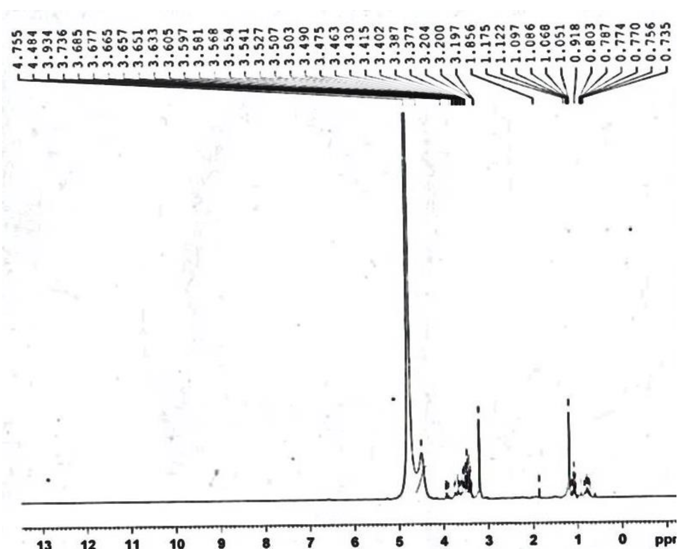

Fig. 3: Proton NMR spectrum for ethanolic rhizome elute, (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

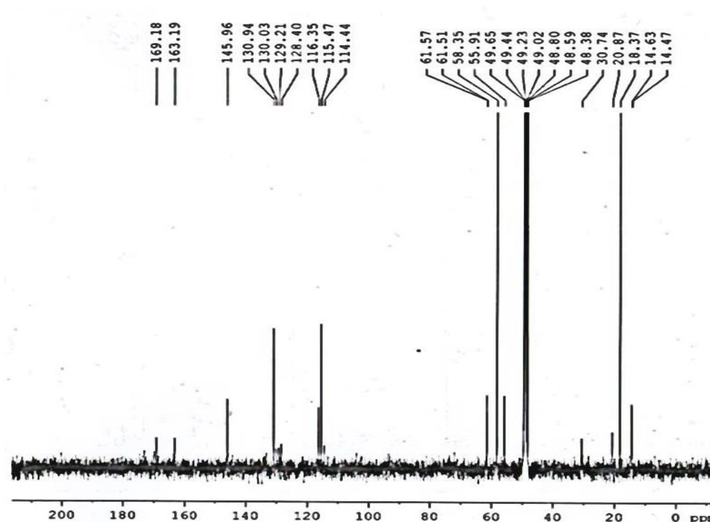

Fig. 4: ^{13}C NMR Spectrum for ethanolic rhizome elute, (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

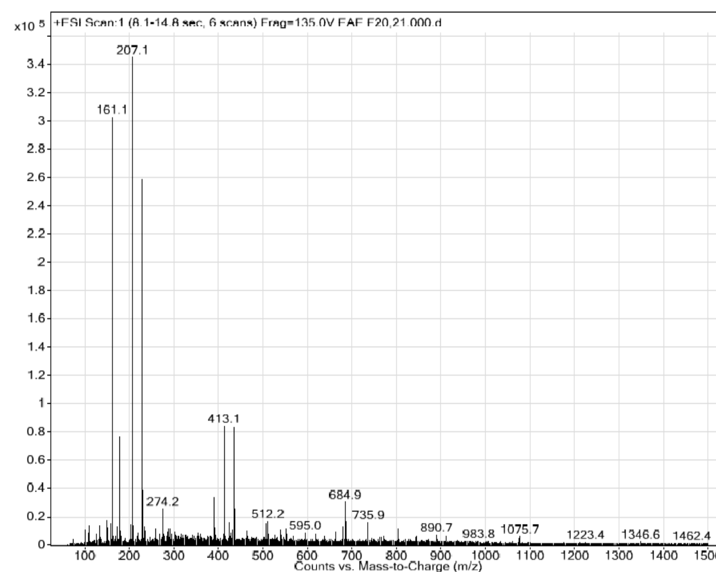

Fig. 5: Mass spectrum for ethanolic rhizome elute, (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galangal*

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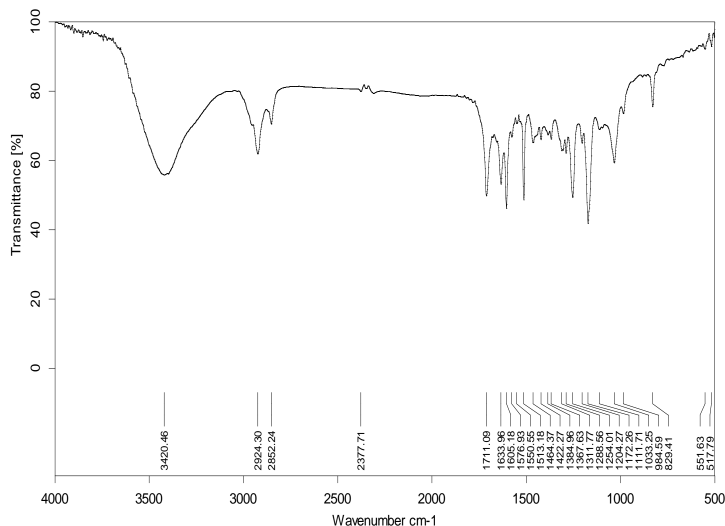

Fig. 6: Infra red spectrum for ethanolic rhizome elute, (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

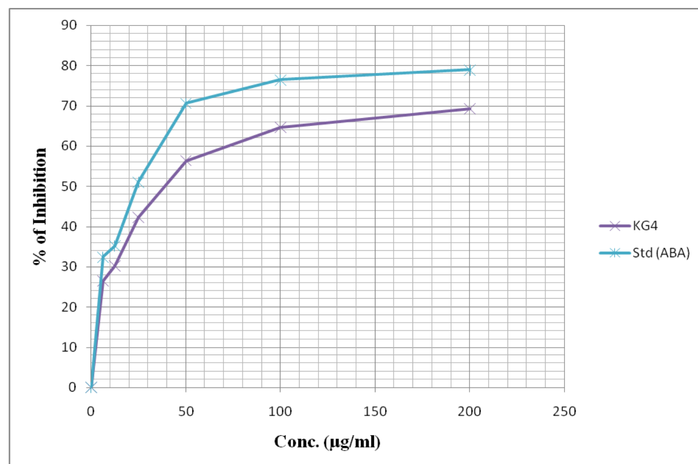

Fig 8: Antioxidant activity (IC_{50}) for rhizome column elute of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galangal*

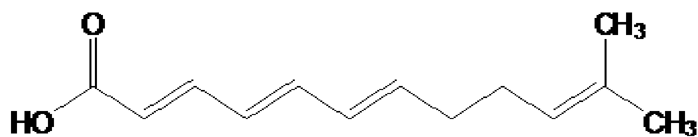

Fig 7: (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid

Table.2 shows the % inhibition readings for antioxidant activity. The IC_{50} for (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid was shown as 39.1 µg/ml and IC_{50} for standard have shown as 24.5µg/ml. (Table 2 &Fig 8)

Table 2: Antioxidant activity of rhizome column elute of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

Concentration	(2E,4E,6E)-11-Methyldodeca-2,4,6,10-tetraenoic acid	Standard (ascorbic acid)
0	0	0
31.25	26.54	32.4
62.5	30.21	35.18
125	42.32	51.02
250	56.43	70.63
500	64.63	76.45

Medicinal plants produce phytochemicals extensively showing different antimicrobial activities. The antimicrobial activities tested with ethanolic rhizome column elute of *K. galanga* on different microbes was shown good results (Table 3). The compound (2E, 4E, 6E)-11-Methyl dodeca-2,4,6,10-tetraenoic acid were shown good antifungal activities (11 to 12 mm) as well as good antibacterial activities (11 to 13 mm). when compared with bacterial standard like Tetracyclin (Antibiotic) shown zone of inhibitions were (10 to 14mm) and for fungi was Fluconazole shown zone of inhibition were (13 to 16 mm) were low.

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Table 3: Antimicrobial activity of rhizome column elute of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga*

Microorganisms	Zone of inhibition in mm (including well size of 8mm) at 50 µg/ml	
	(2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid	Antibiotic
Bacteria		
Bacillus subtilis	11	12
Klebsiella pneumoniae	12	10
Serratia marcescens	11	14
Pseudomonas aeruginosa	11	11
Enterobacter aerogenes	11	10
Escherichia coli	13	10
Fungi		
Aspergillus niger	12	13
Candida albicans	11	16

Note: antibiotic used for bacteria is Tetracyclin and fungi is Fluconazole

The protease inhibition studies for (2E, 4E, 6E)-11-Methyl dodeca-2,4,6,10-tetraenoic acid from *K. galanga* have shown the good inhibition activity with enzymes like protease K and trypsin when compared with chymotrypsin (Table 4 and Fig 9). The standard tetracycline had shown the complete inhibition with proteolytic enzymes and control not shown the inhibition with proteolytic enzymes with X-ray photographic film.

Table 4. IC_{50} values for protease inhibitors (in µM) for (2E, 4E, 6E)-11-Methyl dodeca-2, 4, 6, 10-tetraenoic acid of *K. galanga*

Compound	Protease K	Trypsin	Chymotrypsin
(2E, 4E, 6E)-11-Methyl dodeca-2, 4, 6, 10-tetraenoic acid	31±0. 58	22.67±0.88	36±0.58
Tetracycline(Standard)	22.2±0.66	20.1±0.58	24.6±0.58
Control	0	0	0

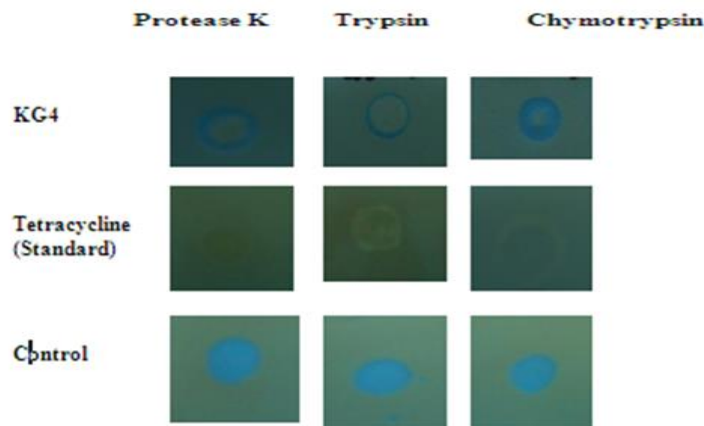

Fig. 9: Protease inhibition assay using X-ray film method

Dose Response of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid of *K. galanga* on MCF-7 (Breast Cancer) cell line shown very less activity 80 µgm/ml when compared with the standard tamoxifen 12.5 µgm/ml (Table 5 and Fig 10).

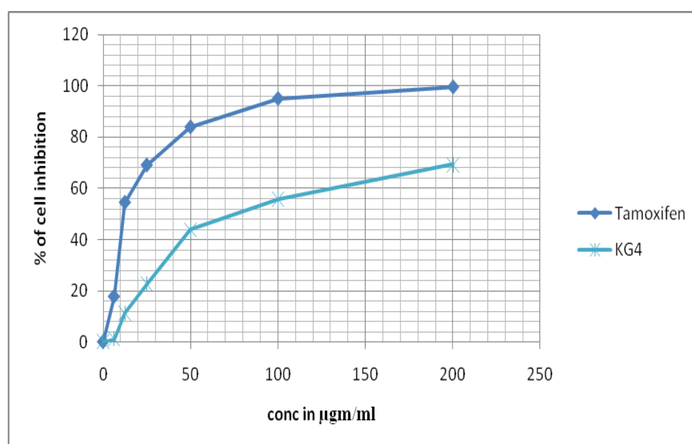

Fig. 10: Antiproliferative activity of (2e, 4e, 6e)-11-methyl dodeca-2,4,6,10-tetraenoic acid of *K. galanga*

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Table 5: Dose Response of (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid column elute of *K. galanga* on MCF-7 (Breast Cancer) cell line

Conc. in (µgm/ml)	% of Cell survival for Tamoxifen	% of Cell inhibition for Tamoxifen	% of cell survival for (2e, 4e, 6e)-11-methyl dodeca-2,4,6, 10-tetraenoic acid	% of cell inhibition for (2e, 4e, 6e)-11-methyl dodeca-2,4,6, 10-tetraenoic acid
0	**100**	**0**	**100**	**0**
6.25	82.3	17.7	98.8	1.2
12.5	45.5	54.5	88.8	11.2
25	30.9	69.1	77.4	22.6
50	16	84	56.1	43.9
100	4.9	95.1	44.2	55.8
200	0.4	99.6	30.7	69.3

DISSCUSSION

Medicinal plants are a great source for economic value in the Indian subcontinent [22]. Nature provides a rich botanical wealth and a huge number of varied types of plants to humans grow in different parts of the country [23]. The several varieties of species are recognized to have medicinal value and the employ of various parts of medicinal plants to heal specific ailments has been established since ancient times [24].

The ethanolic extract of *K. galanga* shown a phytocompound (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid shows antioxidant, antimicrobial, protease inhibition and anti-proliferation activities (Fig. 11).

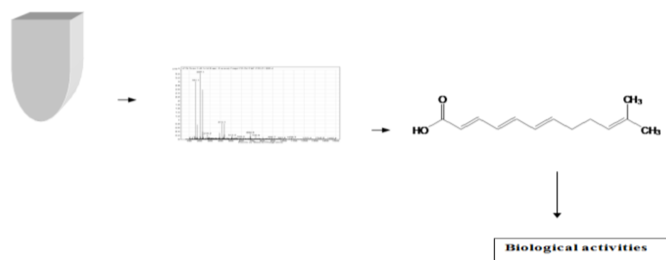

Fig 11: (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10- tetraenoic acid

Herbal medicine is mostly using in developing countries depends as primary healthcare because of better compatibility and cultural suitability with the human body and less side effects [25]. In the present decades multiple drug resistance due to multidrug resistance proteins has developed due to the arbitrary use of antimicrobial drugs that are used in the treatment of infectious microbial and invertebrate diseases [26,27].

The screening for new drugs in plants implies the screening of extracts for the presence of novel compounds and an investigation of their biological activities. The recent innovations in separation and isolation techniques led to a resurgence of interest in their use for the separation of natural products. The purified compound, (2e, 4e, 6e)-11-methyl dodeca-2, 4,6,10-tetraenoic acid was shown good biological activities in the present experimentation.

CONCLUSIONS

The ethanolic extract of *K. galanga* shown a phytocompound (2e, 4e, 6e)-11-methyl dodeca-2, 4, 6, 10-tetraenoic acid shows antioxidant, antimicrobial, protease inhibition and anti-proliferation activities.

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