PHYTOCHEMICAL INVESTIGATION AND ANTI-CANCER, ANTI-INFLAMMATORY AND ANTHELMINTIC ACTIVITY OF METHANOLIC EXTRACT OF LEAVES OF *LEPIDAGATHIS LUTEA* DALZELL

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Abstract

The use of herbal formulations for holistic treatment of lifestyle/ non-communicable diseases and even treating infectious diseases is increasing day by day. Various plants are investigated for their pharmacological potentials. As cancer is the second leading cause of death globally and use of chemical therapeutics suffers from some drawbacks like poor therapeutic performance, multi-drug resistance, and fatal side effects. Finding herbal remedy may overcome these problems, forcing researchers to plant diversity in the treatment of various cancers.

The *Lepidagathis lutea* Dalzell (Acanthaceae) is one of the unexplored endemic plant species to the northern Western Ghats of India. So, the aim of this study is to explore the plant as per modern science. The prepared extract was investigated for broad range phytoceuticalsand tested for itsanti-cancer activity against breast cancer, anti-inflammatory, and anthelmintic activity. The methanolic extract of *Lepidagathis lutea* Dalzell showed the presence of alkaloids, glycosides, carbohydrates, flavonoids, phytosterol, saponins, and tannins. This extract demonstrated considerable anti-inflammatory and anthelmintic activity. Additionally, the extract showed momentous cytotoxicity against breast cancer (MCF-7) cells with IC_{50} value of 46.14 µg/mL.

Thus, *Lepidagathis lutea*Dalzell is observed as promising herbal remedy for treatment of cancer, inflammation, and helminth infections.

Keywords: *Lepidagathislutea* Dalzell, phytoconstituents, anti-inflammatory,anti-cancer, anthelmintic

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INTRODUCTION

The Western Ghats of India contain incomparable levels of plant and animal diversity and endemicity for a continental area. The level of endemicity for about4000 to 5000 plant species recorded in the Ghats; the nearly 650 tree species found in the Western Ghats, 352 (54%) are endemic.[1] Many of these plants are not explored for their medicinal values.

GLOBOCAN 2020 estimated the cancer incidence and mortality worldwide. It has estimated 19.3 million new cancer cases and 10.0 million cancer deaths in 2020. The global cancer burden is expected to rise to 28.4 million cases in 2040, a 47% rise from 2020.[2] It is estimated that almost 2 billion people worldwide are infected with helminths.[3]Chronic inflammatory diseases are also becoming more and more prevalent due to changing lifestyles.[4]

A large number of synthetic medicines are available for the treatment of these diseases. However, side effects, patient non-compliance, are the main hurdles in their use. To overcome these limitations researchers across the globeare placing a strong emphasis on the developing complementary and alternative treatments. Many physicians are also embracing the beneficial aspects of both types of medicine through the practice of integrative medicine; combining appropriate herbal/ ayurvedic medicines and allopathic techniques according to the patient, symptoms, and circumstances.[5]Natural remedies are becoming increasingly popular due to severe financial restrictions and the negative impacts of allopathic therapy.[6, 8-10]According to the World Health Organization, a significant portion of traditional therapies use products made from plant extracts, with roughly 75% to 95% of the population of the world living in developing countries relying mostly on traditional medicines.[7]

Around 110 species of the genus *Lepidagathis* (Acanthaceae) are found across tropical and warm parts of the world. In India, there are 22 species of the genus and one variant in the Western Ghats. The genus *Lepidagathis* has eight distinct variants, 21 of which are native to India.[11]The northern Western Ghats are the only place where *Lepidagathis lutea* Dalzell(LP) is found. It has a woody rootstock and thrives on barren lateritic plateaus in open areas. [12,13]

Some species of wild *Lepidagathis* such as *Lepidagathis hyaline, Lepidagathispungens* Nees reported to show various biological activities like antioxidant, thrombolytic, anxiolytic, anti-depressant, anti-cancer, etc activities. [14,15]However, no any study is reported on the anti-cancer, anti-inflammatory, and anthelmintic activity of *Lepidagathis lutea*.

Therefore, the present research aimed to evaluate the anti-cancer, anti-inflammatory, and anthe-lmintic potential of the methanolic extract of *Lepidagathis lutea*. Further, the development of nanoparticulate system loaded with extract of *Lepidagathis lutea* can be promising to treat cancer, inflammation, and helminth infections, improving the therapeutic performance *via* theminimization of side effects and enhancing patient compliance.

MATERIAL AND METHODS

Plant Collection, Drying, Authentication

Leaves of the plant *Lepidagathis lutea* were collected from Kokan region, India in the month of September. The authentication of plant was done by A.N. Chandore, Botanist at Department of Botanical Laboratories, Abasaheb Marathe Arts and New Commerce, Science College, Rajapur 416702, District Ratnagiri, Maharashtra, India.

Drying of Plant

In the current investigation, after the collection of plant, leaves were separated, and thoroughly cleaned to remove dust and other earthy material. Were dried under the shade, segregated, and pulverized under the mechanical grinder. To protect the plant's phytochemicals, the overheating temperature was purposefully avoided. For the purpose of completely drying out the powder, it was kept in a hot air oven at a temperature not exceeding 60°C.

Extraction of Leaves of *Lepidagathis Lutea* *Microwave Assisted Extraction*

The dry leaves powder was placed in a 250 mL extraction vessel of the microwave system. Methanol in ratio solid: liquid ratio (1:20 gm/mL) was then added. The vessel was inserted inside the microwave cavity and fitted with a condenser. The sample was irradiated with microwaves at 255W, for 20 min of total extraction time. At the end of the irradiation sequence, the solution was left for 1 min before it was filtered using Muslin cloth. And allow the extract to dry for 2-3 days at environmental condition room temperature to get green coloured extract powder.[16,17]

Preliminary Phytochemical Screening

Preliminary analysis of extracts was carried out to identify the presence of various phytoconstituents by employing standard protocols.[18]

Test for alkaloids

- **Dragendorff's test**: 1 mL of Dragendorff's reagent was added 2 mL of extract and observed for orange red precipitate.
- **Mayer's test:** Few drops of Mayer's reagent were added to 1 mL of extract observed for yellowish or white precipitate.
- **Wangner's test:** Few drops of Wagner's reagent were mixed with the 1 mL extract on a watch glass to observe reddish-brown precipitate.
- **Hager's test:** 2 mL of extract was treated with few drops of Hager's reagent; a yellow precipitate indicates the presence of alkaloids.

Test for glycosides

- **Borntrager's test:** 1 mL of extract was treated with 2 mL benzene, shaken properly, and filtered through Whatman's filter paper. The filtrates were then reacted with 2.5 mL of 10% ammonia solution and shaken properly. The presence of pink, red, or violet color in the lower phase indicates presence of anthraquinone glycosides.
- **Keller Killiani test:** 2 mL of extract was mixed with 0.5 mL solution, containing glacial acetic acid and 2-3 drops of ferric chloride. Later, 1 mL of concentrated H_2SO_4 was added along the walls of the test tube. The appearance of deep blue colour at the junction of two liquids indicated the presence of cardiac glycosides.
- **Legal's test:** Few mL of extract was treated with 2 drops of sodium nitroprusside and few mL of pyridine, to which a drop of 20% NaOH was added; formation of pink to red color indicates presence of cardiac glycosides.

Test for flavonoids

- **Ferric chloride test**: About 0.5 g of extract was boiled with distilled water and filtered. To 2 mL of this filtrate, few drops of 10% ferric chloride solution were then added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.
- **Shinoda's test:** 10 drops of dilute HCL and a piece of magnesium were added to 1 mL of extract, observed for deep pink colour.
- **Lead acetate test:** Few mL of extract was dissolved in water and filtered. To 5 ml of each of the filtrate, 3 ml of lead acetate solution was then added. Appearance of a buff-coloured precipitate indicates the presence of flavonoids.
- **Pew's test:** 5 mL of the aqueous solution of the extract was mixed with 0.1 g of metallic zinc and 8 mL of concentrated sulphuric acid. The red colour is indicative of presence of flavonols.
- **Ammonia test:** 3 mL of dilute ammonia was added to 2 mL aqueous filtrate of plant extract; followed by addition of 1 mL concentrated H_2SO_4. Yellow coloration indicated the presence of flavonoids.
- **Alkaline reagent test:** 2 to 3 drops of NaOH were added to 2 mL of extract. Initially, a deep yellow colour appeared but it gradually became colourless by adding few drops of dilute HCL, indicating the presence of flavonoids.
- **Sodium hydroxide test:** Few mL of extract was dissolved in water and filtered; to this 2 ml of the 10% aqueous NaOH was added. Initial yellow colouration turning to colourless on addition of dilute HCL was an indication for the presence of flavonoids.

Test for phenolic compound and tannins

- **Lead tetra acetate test:** 1 mL of lead tetra acetate solution was treated with 0.5 mL of extract, observed for precipitate.
- **Ferric chloride test:** About 0.5 g extract was stirred with about 10 mL of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 mL of the filtrate occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins.

Test for tannins

- **Bromine water test:** Extract was dissolved in 50% alcohol and filtered. The filtrate was reacted with 3-4 drops of bromine water. Appearance of buff color indicated the existence of condensed tannins.
- **Gelatin test:** To a 1% gelatin solution, small quantity of 10% sodium chloride was added, tannins cause precipitation of gelatin.
- **Sodium hydroxide test:** 5 mg of extract was dissolved in 0.5 mL of 20% sulphuric acid solution. Followed by addition of few drops of aqueous sodium hydroxide solution, it turns blue which indicates the presence of phenols.

Test for coumarin

- **Sodium hydroxide test:** 3 mL of 10% NaOH was added to an aqueous plant extract, and yellow colour was observed in positive results.
- **Sodium hydroxide paper test:** 0.5 g of the moistened extract was taken in a test tube. The mouth of the tube was covered with filter paper treated with 1 N NaOH solution. Test

tube was placed for few minutes in boiling water and then the filter paper was removed and examined under the UV light for yellow fluorescence indicated the presence of coumarins.

Test for phytosterol (steroids)

- **Salkowski's test:** Salkowski test: The test extract was shaken with chloroform and concentrated H_2SO_4 was added along the walls of a test tube; and observed for appearance of red colour.
- **Hesse's reaction:** With a few drops of chloroform, a small fraction of the extract was reacted, and an equivalent volume of concentrated sulfuric acid was added to it with the test tube's sides. Appearance of a blood red color confirmed the presence of sterols.

Test for carbohydrates

- **Molish test:** Few drops of alcoholic a-naphthol solution were added to 2 mL of extract. Later, few drops of concentrated H_2SO_4 were added along the walls of test tube. At the junction of two liquids, a violet colour ring appeared, indicating that carbohydrates were present.
- **Benedict's test:** To 5 mL of Benedict's reagent, 8-10 drops extract were added, then heated for five minutes; the resulting dark red precipitate indicated the presence of carbohydrates.
- **Fehling's test:** To 2 mL of extract, an equal volume of Fehling's (A & B) solution was added and heated for five minutes, the resulting red/dark red precipitate indicating the presence of carbohydrates.

Test for saponins

- **Foam test:** A drop of Na_2CO_3 solution was added to 5 mL of extract in a test tube. After vigorous shaking, it was left to rest for five minutes. Foam formation indicated the presence of saponins.

Test for proteins

- **Xanthoproteic test:** The test solution was treated with concentrated nitric acid (Xanthoproteic reagent) solution and boiled properly. Yellow colour indicates presence of protein.
- **Biuret test:** 2 drops of 3% copper sulphate and few drops of 10% NaOH were added to 1 mL of extract, violet or red colouration indicated the presence of proteins.
- **Ninhydrin test**: 2 drops of 0.2% freshly prepared ninhydrin solution added to 1 mL of extract. Purple colour shows the presence of proteins.
- **Millon's test:** 2 mL Millon's reagent was added to the 2 mL extract and heated in a water bath for 5 min. White precipitate, which turns red after heating, indicates the presence of proteins.

Test for starch

- **Iodine test:** 2 mL of iodine solution with potassium iodine were added to 2 mL of test extract and observed for blue colour.

Test for triterpenoids

- **Horizon test:** 2 mL of trichloroacetic acid was added to 1 mL of extract and observed for red precipitate.

Pharmacological Evaluation

In vitro Anthelmintic Activity

Indian adult earthworms (*Pheretimaposthuma*) measuring 7-9 cm in length and 0.70-0.90 g in weight were used to test the anthelmintic activity of *Lepidagathis lutea* leaf extracts. They are divided into six groups each containing six earthworms were placed in 5 different concentrations of the test while one group for standard as piperazine citrate. Saline solution was used to obtain desired drug concentrations. The duration of paralysis and death was observed. A mean ± SEM of six animals in each group was used to express all the results. [19,20]

In vitro Anti-inflammatory Activity

Proteins lose their structural integrity as a result of the presence of other substances, stress from the environment, or heat, which renders them ineffective for biological purpose and is termed protein denaturation. As a result, the denaturation of tissue proteins is understood to be a sign of inflammation. With final concentrations of 20, 40, 80, 160, and 320 µg/mL, the reaction mixture, which had a volume of 10 mL, comprises 0.4mL of fresh hen's egg albumin, 5.6 mL of phosphate buffered saline (PBS, pH 6.4), and 4 mL of extract. Control was an equivalent volume of phosphate buffer (pH 6.4). The mixtures were then heated for 5 minutes at 70°C after 15 minutes of incubation at (372) °C in a BOD incubator. After cooling, their absorbance at 660 nm using a vehicle as a blank was measured by UV spectrophotometer. Final concentrations of 100, 200, 300, and 400 µg/mL of diclofenac sodium were used as the reference drug. [21,22]Protein

denaturation inhibition was calculated using the formula below:

$$\% \, Inhibition = 100 \, \text{X} \, \frac{Vt}{(Vc - 1)}$$

Where, Vt = absorbance of test sample
Vc = absorbance of control

In vitro Anti-cancer Activity

This study made use of the MCF7 human tumor breast cell line. The cytotoxic activity of the extract was measured *in vitro* for in comparison to standard drug 5 fluorouracil (5-FU). Cells were incubated at a concentration of $1x10^4$ cells /mL in culture medium for 24 h at 37°C and 5 % CO_2. Then it was seeded at a concentration 100 µL and 10, 40, 100 µg/mL of samples into a microplate respectively placed in CO_2 incubator for 48 h (37°C, 5 % CO_2). The medium is then removed, add 20 µL MTT to each well, and incubated for 4 h. Under a microscope, examine the wells for the production of formazan crystals. The medium is removed and 200 µL DMSO is added to each well incubated at 37°C. Triplicate samples were analyzed by measuring the absorbance of each sample with amicroplate reader at a wavelength of 550 nm. Based on 100% vitality, untreated cells are referred to as a control. [23,24]

$$\% \, Inhibition = \frac{(\text{Control} - \text{Test})}{\text{Control}} \text{X}100$$

Statistical Analysis

One-way analysis of variance (ANOVA) was used to statistically evaluate the experimental data, and then the Dunnett test was performed. The data was presented graphically using GraphPad Prism 9.4.1 software. Mean ± SEM was used to express the data. A difference was deemed significant at $p< 0.05$.

RESULT AND DISCUSSION

Preliminary Phytochemical Screening

Preliminary phytochemical investigation of *Lepidagathislutea* methanolic extract has revealed the presence of alkaloids, glycosides, carbohydrates, flavonoids, phytosterol, saponins, and tannins, as depicted in Table 1. Some of these phytoconstituents may be responsible for anti-cancer, anti-inflammatory, and anthelmintic properties.

Table 1: Phytochemical investigation of methanolic extracts of *Lepidagathislutea*

Chemical test	Result	Chemical test	Result
Test for alkaloids		*Test for coumarin*	
a) Dragendorff's test	+	a) Sodium hydroxide test	−
b) Mayer's test	+	b) Sodium hydroxide paper test	−
c) Wagner's test	+	*Test for phytosterol*	
d) Hager's test	+	a) Salkowski's test	+
Test for glycosides		b) Hesse's test	+
a) Borntrager's test	+	c) Sulphur test	+
b) Killer Killanitest	+	*Test for carbohydrates*	
c) Legal's test	+	a) Molisch test	+
Test for flavonoids		b) Benedict's test	+
a) Ferric chloride test	+	*Test for saponins*	
b) Shinoda's test	+	a) Foam test	+
c) Lead acetate test	+	*Test for amino acids and proteins*	
d) Pew's test	+	a) Xanthoproteic test	−
e) Ammonia test	+	b) Biuret test	−
e) Conc. H_2SO_4 test	+	c) Millon's test	−
		Test for tannins	
Test for phenolic compound		a) Bromine water test	+
a) Iodine test	+	b) Lead sub acetate test	+
b) Lead acetate	+	c) Gelatin test	+
c) Ferric chloride test	+	d) 10% sodium hydroxide test	+

Pharmacological Evaluation

In vitro Anthelmintic Activity

The anthelmintic activity of the extract of *Lepidagathis lutea* was determined and compared with the standard drug piperazine (Table 2, Figure 1). The extract demonstrated dose-dependent anthelmintic activity. The extract (20mg/mL) caused rapid paralysis at 19±0.14 min and death at 24.1±0.19 min, respectively. On the other hand, the standard drug piperazine citrate showed paralysis at 48.8±0.273 min and death after 53.9±0.296 min. Thus, the extract showed potent anthelmintic activity when compared to the piperazine.

Table 2: Anthelmintic activity of *Lepidagathis lutea* methanolic extract, indicated by paralysis and death time

Concentration (mg/mL)	Time taken for paralysis (P) and death (D)	
	Paralysis time (Min)	Death time (Min)
Control (DMSO + Saline water)	-	-
2	45 ± 0.0923	47.7 ± 0.273
5	39.8 ± 0.205	44.2 ± 0.190
10	34 ± 0.167	38.2 ± 0.144
15	26.1 ± 0.190	35.1 ± 0.173
20	19 ± 0.140	24.1 ± 0.197
Std. (piperazine citrate)	48.8 ± 0.273	53.9 ± 0.296

Results are expressed as mean ± SEM; $p<0.05$ considered as significant when compared with control (one-way ANOVA followed by Dunnett's Test)

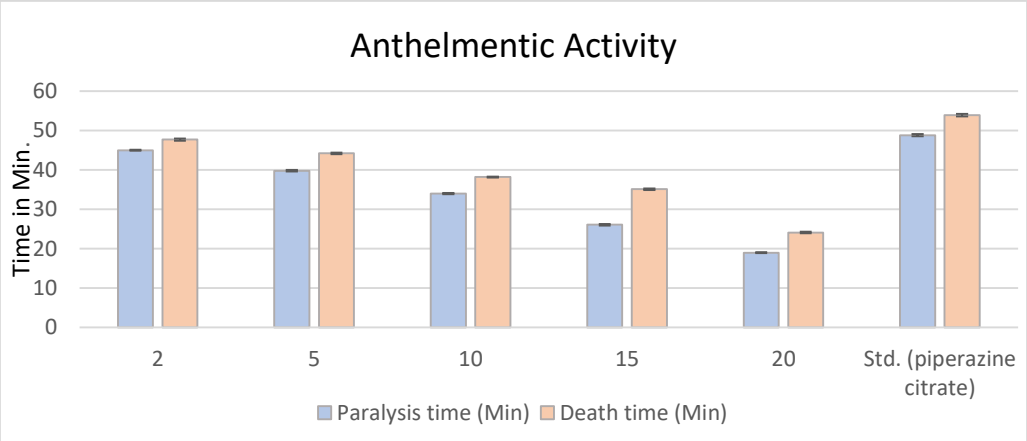

Fig 1: Anthelmintic activity*Lepidagathis lutea* methanolicextract against *Pheretimaposthuma* indicated by paralysis and death time

Anti-inflammatory Activity

The *in vitro* anti-inflammatory activity of *Lepidagathis lutea* extract is evaluated based on its ability to inhibit protein denaturation and compared with diclofenac (Table 3). The extract showed significantly higher protein denaturation inhibition than diclofenac sodium. The 27.1% inhibition of albumin denaturation was observed with extract at a concentration of only (20 µg/mL). In contrast, diclofenac inhibited 27% of albumin denaturation at a concentration of 100 µg/ mL. These obtained results were further confirmed by their IC_{50} values. The extract showed a very low IC_{50} value (41.22 µg/ mL) than diclofenac sodium 204 µg/mL, as shown in Figure 2. Thus, the obtained results revealed the potent anti-inflammatory activity of the extract.

Table 3: Anti-inflammatory potential of methanolic extract of *Lepidagathis lutea*in comparison with standard drug (diclofenac sodium) measured by protein denaturation

Sr. No.	Concentration (µg/mL)	Percentage inhibition (%)	IC $_{50}$ (µg/mL)
Effect of extract on protein denaturation	20	27.1 ± 0.227	41.22
	40	45.3 ± 0.314	
	80	78.6 ± 0.394	
	160	116 ± 0.573	
	320	160 ± 0.114	
Effect of diclofenac sodium on protein denaturation	100	27 ± 0.299	204
	200	47.3 ± 0.178	
	300	74.6 ± 0.251	
	400	92 ± 0.290	

Results are expressed as mean ± SEM; $p<0.05$ considered as significant when compared with control (one-way ANOVA followed by Dunnett's Test)

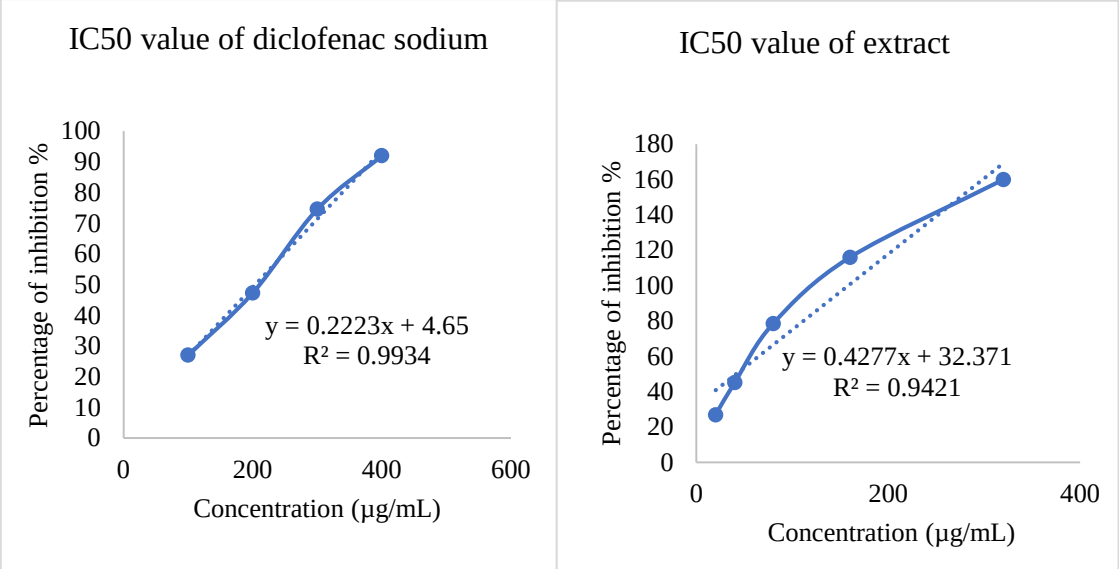

Fig 2: Anti-inflammatory activity methanolic extract of *Lepidagathis lutea*: IC_{50} values of extract and reference drug

Anti-cancer Activity

The anti-canceractivity of the extract was determined by using MTT dye assay against breast cancer (MCF-7) cells and was compared with 5-FU. Both extract and 5-FU showed cytotoxicity in a dose-dependent manner (Table No. 4). The extract showed more cytotoxicity (low IC_{50}: 46.14 µg/mL) than (IC_{50}: 50.18 µg/mL). No significant difference was observed in the cytotoxicity of the extract and 5-FU. Thus, the cytotoxicity study results revealed the anti-cancer potential of activity the extract against breast cancer (MCF-7) cells.

Table 4: Effects of methanolic extract of*Lepidagathis lutea*of against MCF-7 cell line by MTT assay, in comparison with 5-FU as standard

Sample	Concentration (µg/ml)	Absorbance	Mean	% Inhibition	IC_{50} (µg/ml)
Control	-	0.846	0.846	-	-
Standard (5-FU)	10	0.618 0.347 0.598	0.521±0.087	38.41±0.237	50.18
	40	0.456 0.426 0.378	0.42±0.023	50.35±0.424	
	100	0.319 0.326 0.342	0.329±0.007	61.11±0.107	
Methanolic extract of *Lepidagathis lutea* leaves	10	0.468 0.495 0.501	0.488±0.010	42.31±0.315	46.14
	40	0.390 0.445 0.454	0.430±0.02	49.29±0.222	
	100	0.315 0.346 0.335	0.332±0.009	60.75±0.176	

Results are expressed as mean ± SEM; $p<0.05$ considered as significant when compared with control (one-way ANOVA followed by Dunnett's Test)

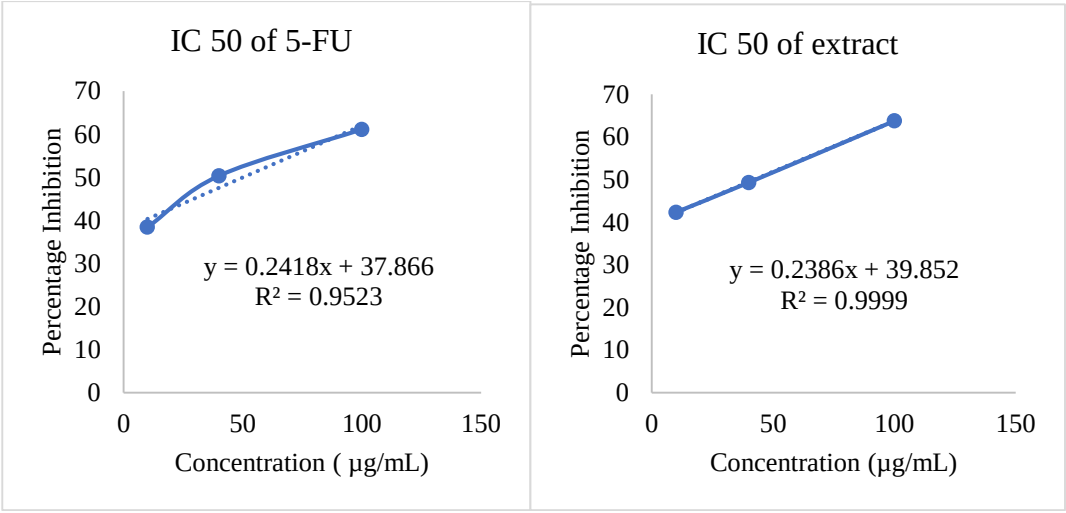

Fig. 3: Graph showing IC_{50} values of extract and reference drug (5-FU) for anti-cancer activity

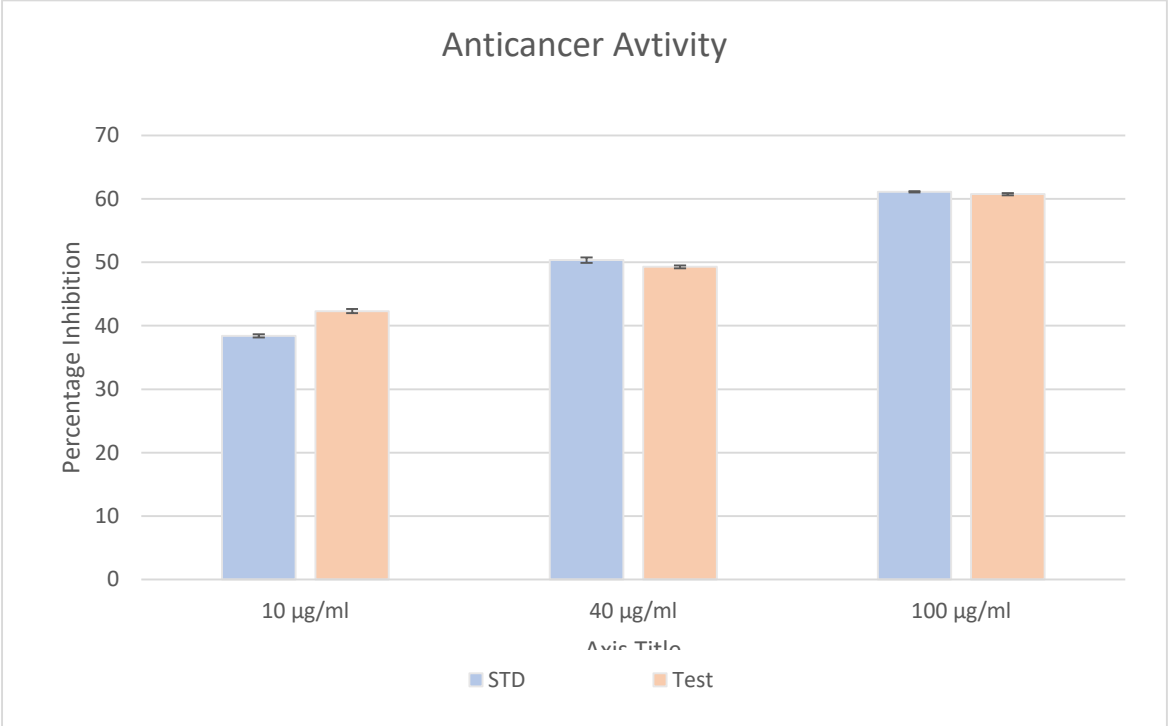

Fig 4: Anticancer activity *Lepidagathis lutea* methanolic extract against MCF-7 cell line using MTT assay

CONCLUSION

Lepidagathis lutea (Acanthaceae)is an endemic species from Western Ghats of India. Karyomorphological analysis of the plant is previously reported with not much exploration for its phytoceuticals and medicinal uses. It is reported to be antioxidant and larvicidal too. In this study, methanolic extract of *Lepidagathis lutea*was successfully investigated for the presence of alkaloids, flavonoids, phenolics and tannins, glycosides and sterols. The extract elicited significant anti-cancer, anti-inflammatory and anthelmintic activity.

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