JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2025/46250
RESEARCH ARTICLE

Bioactive Profiling of *Kaempferia galanga* Rhizome Extract: An *In Vitro* Study of its Antibacterial, Anticancer and Apoptotic Activity

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Abstract

Background: Given the emergence of resistance to conventional treatment of illnesses such as cancer and chronic infections, identifying other natural products cannot be overemphasised to help combat this problem. The rhizome of *Kaempferia galanga,* an aromatic plant predominantly found in South Asian regions, has diverse biological properties that contribute to its potential therapeutic activity. **Aim:** This study aimed to characterise biochemical constituents and evaluate biological applications using the rhizome of *Kaempferia galanga* extract. **Methods:** Aqueous and ethanol extract of rhizome subjected to phytochemical screening, Thin Layer Chromatography (TLC), *In vitro* antioxidant tests, *in-vitro* anti-inflammatory tests, Gas Chromatography-Mass Spectrometry (GC-MS) and antibacterial was checked with well-diffusion assay. MTT assay using different concentrations of ethanol extract on the HeLa cell line was done to study its anti-cancer effect and apoptosis study was done by flow cytometry. **Results:** Phytochemical screening and Thin Layer Chromatography (TLC) of the rhizome extracts showed the presence of bioactive components. *In-vitro* tests indicated that the rhizome has antioxidant and anti-inflammatory properties. GC-MS of ethanol extract shows the presence of many bioactive constituents with therapeutic benefits, including the well-described Ethyl-p-methoxycinnamate. The antibacterial zones of inhibition ranged from 12mm to 17mm. In the MTT assay, there was an increase in the percentage of cell death with increasing concentration of extract and the IC_{50} value was found to be 25.04µg/mL. The percentage of total apoptotic cells was 24.63%. **Conclusion:** This study has found that *K. galanga* rhizome has many bioactive compounds that help to provide its antioxidant, anti-inflammatory, antibacterial and anticancer activity (*In vitro* study). Hence, further *in-vivo* studies may provide insight into whether the rhizome extracts of *K. galanga* can be used as an adjunct to available traditional treatment of chronic diseases.

Major Findings: *Kaempferia galanga* rhizome extract has been shown to be an effective alternative antimicrobial and anticancer compound, with studies demonstrating its ability to enhance cell apoptosis and improve cell inhibition rates supporting its therapeutic use in traditional medicine system.

Keywords: Antioxidant, Apoptosis, GC-MS, *Kaempferia galanga*, MTT Assay

1. Introduction

Kaempferia galanga, commonly referred to as aromatic ginger, belongs to the family *Zingiberaceae* and is commonly distributed in Southeast Asian regions[1]. The perennial herb has a characteristic fragrant underground rhizome that has been used in some Asian cuisines and as home remedies to cure common ailments since ancient times[2]. It is a component in various *Ayurvedic* formulations and is also used by certain tribal communities for treating cough, indigestion, rheumatism and other painful conditions, indicating its role in reducing inflammation[3].

In an *in vitro* study using different solvent systems it was observed that rhizome distillate of *K. galanga* has a dose-dependent antioxidant activity. The essential oil extract of rhizome containing high amounts of the active chemical component namely Ethyl-p-methoxycinnamate, has potent anti-inflammatory properties and antimicrobial activity, among other

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Article Received on: 05.10.2024 Revised on: 20.01.2025 Accepted on: 28.02.2025

effects such as antidiabetic and anticholinesterase properties[5]. The rhizome oil has been suggested to be used as a UV-protective compound that may protect against inflammation of the skin[6]. A study done to evaluate wound healing in chemically-induced oral ulcers in Wistar rats showed that *K. galanga* extracts hastened the healing process[7].

Studies have shown that *K. galanga* has significant anti-microbial activity[8,9]. The rhizome extract has antiviral effects on Hepatitis E Virus (HEV) by preventing the binding of HEV with specific receptors[10]. A study done on the CL-6 cell line concluded that the bioactive component of *K. galanga* had moderate anticancer activity[11]. *K. galanga* extract has been shown to have an anti-proliferative effect on Mitochondrial Transcriptional Factor A (TFAM) tumour cells[12].

The inherent properties of anti-inflammatory, antioxidant constituents of *K. galanga*, with the additional antimicrobial efficacy, make it an ideal candidate to be used as an adjunct to the conventional therapeutic options available for the treatment of various inflammatory, infective and neoplastic states. This study aimed to do the phytochemical analysis and characterisation by TLC and GC-MS and also, to evaluate the antioxidant, antibacterial, anti-inflammatory, anticancer and apoptotic activity of *K. galanga* rhizome extract by *in vitro* methods.

2. Materials and Methods

2.1 Plant Sample Collection

Kaempferia galanga plant was procured from a nursery in Thrissur, Kerala, India and identified by the Professor and Head Department of Biotechnology, Alva's College Moodubidire, Karnataka, India. The collected plant species were carefully transported to the laboratory for further procedures.

2.2 Chemicals Used in the Study

The chemicals used in this study were obtained from Isochem Laboratories (India), Rankem Chemicals (India), Nice Chemicals (India), HIMEDIA (India), Spectrum Chemicals (India), and Fisher Scientific Chemicals (India) supplied the chemicals and reagents required for the research. All these chemicals were of analytical grade.

2.3 Preparation of Rhizome Extract

The rhizome of the collected sample was separated, washed thoroughly followed by cut into small pieces. Then the rhizome pieces were dried completely in the shade and powdered using a mortar and pestle. 1% extracts were prepared by mixing the powdered rhizome with solvents namely distilled water and ethanol separately. The mixtures were kept in a rotary shaker for incubation at 60 - 70 RPM, at 40°C for 24 hours and then filtered[13]. The obtained aqueous and ethanol extract was taken for further analysis.

2.4 Phytochemical Analysis

The aqueous and ethanol extracts of the sample were subjected to standard tests[14,15] to detect the presence of major bioactive compounds such as steroids, terpenoids, phenols, sugars, saponins, flavonoids, quinines, proteins and alkaloids.

2.5 Estimation of Antioxidant Activities

2.5.1 Phosphomolybdenum Method

The phosphomolybdenum method is a commonly used method to measure the antioxidant activity of extracts. A 0.5mL reaction mixture (0.6 M H_2SO_4, 28 mM sodium phosphate, 4mM ammonium molybdate) was added to 0.5mL of the rhizome extracts. After mixing it was kept in a water bath at 50°C for 1.30 hours. The absorbance of the sample was taken at 695nm (LT 291 Labtronics microprocessor-spectrophotometer)[16]. The formula provided was used to calculate the antioxidant capacity:

$$\text{Percentage of Antioxidant effect} = \frac{\text{Absorbance Control - Absorbance of Sample}}{\text{Absorbance Control}} \times 100$$

2.5.2 DPPH Method

2,2-Diphenyl-1-picrylhydrazyl (DPPH), assay was tested to assess the antioxidant activity of the rhizome extracts. 0.2mL of 0.1 N DPPH solution was mixed with 0.5mL of sample and incubated for 5 minutes. 0.4mL 50mM Tris Hydrochloric Acid (HCl) was added and kept for 30 minutes. The OD was taken using a spectrophotometer at (LT 291 Labtronics microprocessor) 517nm[16].

2.6 Anti-inflammatory Activities

2.6.1 Protein Denaturation Method

Tissue protein denaturation is a marker for inflammation and therefore, any agent that prevents protein denaturation possesses the possibility to use it as an anti-inflammatory drug. Inhibition of protein denaturation, one of the *in vitro* models for determining anti-inflammatory activity was used in this study[17]. 1mL sample, 0.5mL of diclofenac and 3mL of phosphate buffer solution mixed with 1mL of egg albumin and incubated for 20 minutes at 37°C and again at 90°C for 2 minutes. The mixture was cooled and the absorbance was measured at 660nm using a spectrophotometer. The percentage of anti-inflammatory activity was calculated as:

$$\text{Anti-inflammatory\%} = \frac{AC - AT}{AC} \times 100$$

(AC- Absorbance of Control, AT- Absorbance of Test)

2.6.2 Trypsin method

The trypsin inhibitory method, a proteinase inhibitory assay, was carried out by adding 0.06mg of Trypsin and 1mL of 20mM Tris HCl to 1mL of sample and incubated at 37°C for 5 minutes. Then, 0.8% casein solution was added and incubated for 20 minutes. This was followed by adding 2mL of 70% perchloric acid and centrifuged at 5000rpm[18]. The clear solution was used to measure at 210nm using an Ultra Violet (UV) spectrophotometer. The percentage of anti-inflammatory activity was calculated as:

$$\text{Percentage of Anti} - \text{Inflamatory} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

2.7 Thin Layer Chromatography

TLC was performed on rhizome extracts using Merck TLC Silica plates on aluminium sheets. The mobile phase consisted of a mixture of Toluene: Ethyl acetate and formic acid at a ratio of 5:4:0.2. 0.5µl of sample was spotted on TLC plates using capillary tubes and the plates were then placed in a TLC chamber containing the mobile phase, ensuring that the sample spots were positioned above the level of the mobile phase, and the chamber was covered with a lid. After development, the plates were removed and allowed to dry. The spots were visualized by exposing the plates to iodine vapour, and the corresponding R_f values were calculated[19].

2.8 Gas Chromatography-Mass Spectrometry

GC-MS analysis is a superior, highly accurate test to characterise the various phytochemical constituents in plant extracts and to correlate their biological function, as the latest instruments are coupled to the mass spectral database[20]. The ethanol extract was subjected to GC-MS analysis using Agilent GC-MS Spectrometer Model CH-GCMSMS02 with settings as per protocol[16]. The gas carrier was Helium and Ethanol was used as diluent. The total run time was 38 minutes and the scan range was 30-900 m/z. Data acquisition was done using Mass Hunter Acquisition software for GC-MS systems.

2.9 Tests for Antibacterial Efficacy

Antibacterial efficacy was tested by well diffusion method[21] using *Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli*. The bacterial cultures were swabbed and the extracts (aqueous and ethanol) were added to the wells that were cut using a cork borer. Antibiotic Azithromycin was taken as guaranteed control and Dimethyl Sulfoxide (DMSO) was preferred as negative control. The plates were incubated for 24 hours at 37°C and the results were noted in mm.

2.10 Anticancer Activity of the Extract

The anticancer effect of ethanolic solution of *Kaempferia galanga* on the HeLa cell line was tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In the MTT assay, yellow substrate MTT is converted to formazan (purple product) by active mitochondria and the colourimetric quantification by spectrophotometry can be correlated with cell number[22]. HeLa cell line was seeded with Dulbecco's Modified Eagle Medium (DMEM) and grown in a CO_2 incubator (CO_2 incubator contains - 80% humidity, 5% CO_2 and 37° temperature). In a sterile 96-well plate, 100µL of the cell line was mixed with different concentrations (5, 10, 15, 20, 25 µL) of samples and 100µL of the cell line was taken as control. This was incubated in a CO_2 incubator for another 24

hours. It was then washed (trypsin and DMSO) and 20 µL MTT dye was added and incubated for 4 hours. The percentage of cell death was calculated after taking the OD value at 570nm using an ELISA reader.

2.11 Apoptosis Study

Apoptotic activity was determined by Flowcytometry analysis using Annexin V which tends to bind with Phosphatidylserine exposed during apoptosis[23]. HeLa cell line was cultured as 0.5×10^6 cells/2mL in a 6-well plate and incubated in a CO_2 incubator at 37°C for 24 hours. After removal of the medium, cell lines were mixed with ethanol extract and incubated for 24 hrs. After incubation, PBS wash was done followed by Trypsin solution (200µL) was added and kept at 37°C for 4 minutes. 2mL culture medium was added and the cells were harvested into polystyrene tubes. The tubes were subjected to centrifugation at 300 x g at 25°C for 5 minutes. The supernatant was removed, followed by Phosphate-Buffered Saline (PBS) wash and removal. FITC Annexin V (5µL) was added to this, mixed in a vortex and incubated at 25°C for 15 minutes. 400µL of 1X Annexin binding buffer and 5µL of PI were added and mixed in the vortex. The obtained mixtures were analysed by flow cytometry.

3. Results

3.1 Phytochemical Analysis

The presence of compounds such as flavonoids, phenols, and steroids is responsible for different biological applications of medicinal plant extracts. The results of various phytochemicals in *K. galanga* are summarised in Table 1.

The aqueous sample showed the presence of steroids, flavonoids proteins and alkaloids and was negative for phenol. Tests done on the ethanol extract indicated the presence of alkaloids, flavonoids, quinines and steroids, whereas phenol, saponins and proteins were not present.

3.2 Antioxidant Activity

In vitro antioxidant activity was done for both the extracts (aqueous and ethanol) by phosphomolybdenum method and DPPH assay (Figure 1). The antioxidant activity by the phosphomolybdenum method was observed to be 156µg/mL in aqueous extract and 136 µg/mL in ethanol extract. By DPPH assay, the antioxidant activity for aqueous and ethanol was 84 µg/mL and 96 µg/mL respectively.

3.3 Anti-inflammatory Activity

The results of *in vitro* anti-inflammatory studies by protein denaturation and Trypsin method done on aqueous and ethanol extracts are shown in Figure 2. The percentage of anti-inflammatory activity by the

Table 1. Phytochemical analysis of *K. galanga* rhizome extract

Test	Aqueous	Ethanol
Alkaloid	+	+
Terpenoids	+	+
Phenol	-	-
Sugar	+	+
Saponins	+	-
Flavanoids	+	+
Quinines	+	+
Proteins	+	-
Steroids	+	+

+ denoting presence of compound; - denoting absence of compound

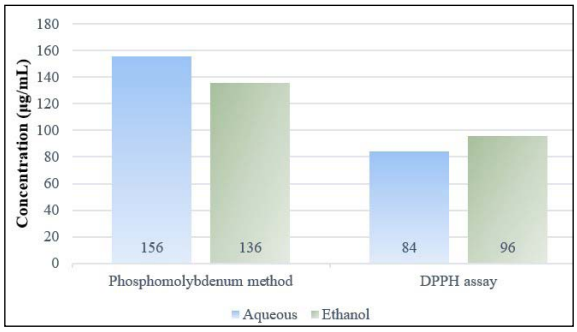


Figure 1. Antioxidant activity of *K. galanga* extract.

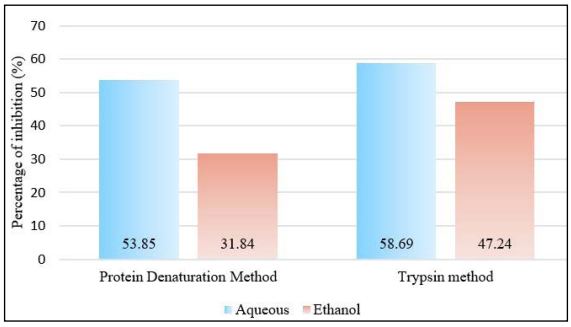


Figure 2. Anti-inflammatory of *K. galanga* extracts.

Protein denaturation method and Trypsin method was found to be 53.85% and 58.69% in aqueous extract respectively. The anti-inflammatory activity in ethanol extract was 31.84% and 47.24% by the Protein denaturation method and Trypsin method respectively.

3.4 Thin Layer Chromatography

TLC was performed for the aqueous and ethanol extracts and the different component bands were visualized. For the aqueous extract, C1 and C2 with R_f values of 0.322 and 0.596 for ethanol extract C1, C2, C3 and C4 with R_f values of 0.316, 0.45, 0.516, 0.6 were obtained respectively.

3.5 Gas Chromatography-Mass Spectrometry

GC-MS study revealed the showing various bioactive compounds. The names and chemical formulas of some of the compounds along with their biological activity are summarized in Table 2.

3.6 Antibacterial Activity

The antibacterial efficacy was studied by the well diffusion method (Figure 3). The zones of inhibition from 12mm to 17 mm and the maximum zone of inhibition (17mm) were obtained for the aqueous extract of *K. galanga* on *E. coli*. Many bioactive compounds found in the extract, as determined by GC-MS analysis (Table 2), have antimicrobial properties.

Table 2. Chemical name, formula and biological activity of a few compounds identified by GC-MS analysis of ethanol extract of *K. galanga* rhizome

S. No.	Compound name	Formula	Biological activity
1	2-Propanol, 1-methoxy-	$C_4H_{10}O_2$	Antioxidant
2	1H-Tetrazole	CH_2N_4	Antimicrobial, antifungal, analgesic, anti-inflammatory
3	Silanediol, dimethyl-	$C_2H_8O_2Si$	Antimicrobial, anti-inflammatory
4	Guanidine	CH_5N_3	Anticancer and antimicrobial
5	3,5-Dithiahexanol 5,5-dioxide	$C_4H_{10}O_3S_2$	Anticancer and antimicrobial
6	Triethylsilanol	$C_6H_{16}OSi$	Antimicrobial
7	5-Iodopyrid-2(1H)-thione	C_5H_4INS	Anticancer
8	Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	Antioxidant, anticancer and antimicrobial
9	3-Carene	$C_{10}H_{16}$	Anti-inflammatory, antimicrobial and anticancer
10	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	Antioxidant
11	Phloroglucinaldehyde, tris(trimethylsilyl) ether	$C_{16}H_{30}O_4Si_3$	Antioxidant and antimicrobial
12	Bicyclo 2.2.1heptan-2-ol, 1,7,7-trimethyl-, 1S-endo	$C_{10}H_{18}O$	Wound healing
13	alpha-Terpineol	$C_{10}H_{18}O$	Antioxidant, anti-inflammatory, antimicrobial, anti-proliferative and anticancer
14	2,4-Quinolinediol	$C_9H_7NO_2$	Antimicrobial and anticancer
15	2-Hydroxyfluorenone, acetate	$C_{15}H_{10}O_3$	Antibacterial
16	Ethyl p-methoxycinnamate	$C_{12}H_{14}O_3$	Wound healing, anti-inflammatory, anti-angiogenic, anticancer and anti-proliferative
17	Phthalic acid, cyclohexylpentyl ester	$C_{19}H_{26}O_4$	Anti-inflammatory
18	Octadecane	$C_{18}H_{38}$	Antimicrobial and anticancer
19	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	Anti-inflammatory essential oil
20	Squalene	$C_{30}H_{50}$	Wound healing, antimicrobial

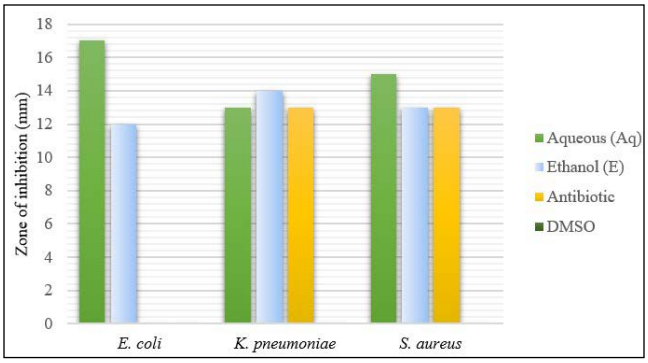


Figure 3. Antibacterial activity of *K. galanga* extracts.

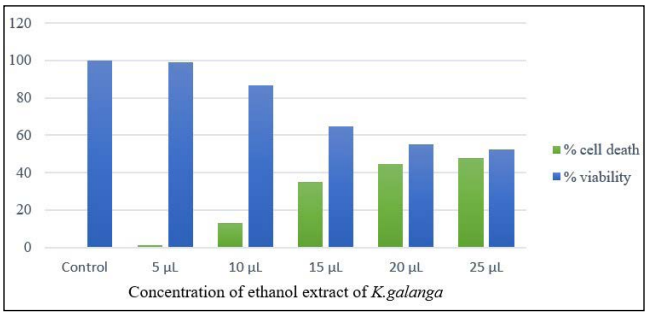


Figure 4. MTT assay on HeLa cell line treated with *K. galanga* extract.

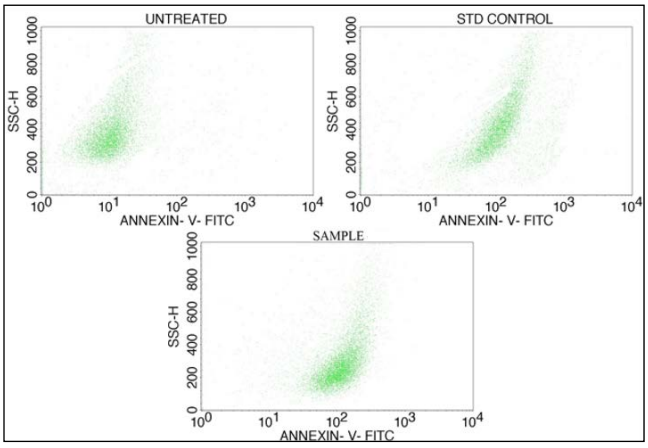


Figure 5. Flow cytometry results showing apoptotic activity of untreated, standard control and ethanol extract samples of *K. galanga*.

3.7 Anticancer Activity

MTT assay to detect the anticancer effect was done on the HeLa cancer cell line using different concentrations of ethanol extracts of *K. galanga*. The absorbance values obtained were used to calculate the percentage of cell death and cell viability and the results are presented in Figure 4.

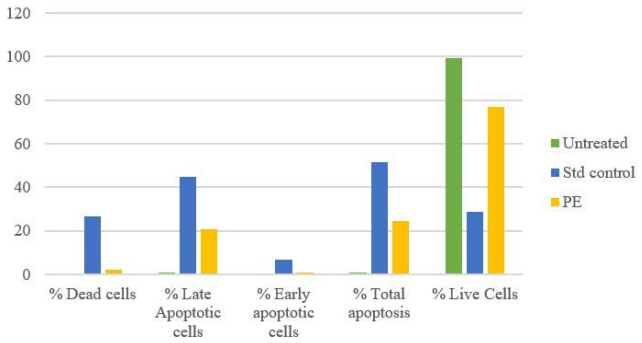


Figure 6. Flow cytometry analysis showing Percentage of apoptotic cells in untreated, standard control, *K. galanga* rhizome extract (PE) treated HeLa cell.

3.8 Apoptotic Study

The apoptotic activity of the ethanol extract on HeLa cells is shown in Figures 5 and 6. The percentage of total apoptotic cells was observed to be 24.63%. The percentage of cells in early and late apoptotic stages were 0.86% and 20.86 % respectively.

4. Discussion

Kaempferia galanga, a member of the *Zingiberaceae* family, is a promising medicinal plant known for its aromatic rhizome. In traditional medicine across Asian countries, local practitioners commonly utilise this plant. Phytochemical analysis of the sample showed its medicinal importance and a previous study stated that saponins, phenolics and flavonoids were present while proteins were absent in both aqueous and ethanol extracts of *K. galanga*[1]. In the same study, it was shown that the alkaloids and carbohydrates were present in ethanol extract but absent in aqueous extract. The phenol content varies with the plants grown in different altitudes[25]. Besides, a higher temperature applied during the extraction process reduced the amount of phenol[9]. Compounds like flavonoids, phenols and steroids contribute to the diverse biological applications of medicinal plant extracts[24]. Findings of Balouiri *et al.*, concluded that *K. galanga* extract had maximum anti-oxidant activity at 500 µg/mL by DPPH assay[4]. In another study, it was found that the inhibitory concentration of the ethanol of *K. galanga* was 58.79 µg/mL when compared with ascorbic acid[1]. Ali *et al.*, stated that heat treatment on antioxidant content was

tested and observed loss of anti-oxidant content on rhizome extract when it heats above 80°C[1].

Anti-inflammatory activity of *K. galanga* by Protein Denaturation Inhibition method, it was observed that the anti-inflammatory effect had a dose-dependent pattern (25% to 81%) as the concentration of the extract increased[17]. In the same study, for the aqueous extract, the anti-inflammatory effect ranged from 20% to 65% and for the chloroform extract, the activity was 30% to 85%. Ethyl-p-methoxycinnamate is a potent anti-inflammatory constituent in *K. galanga* extracts and inhibits COX, TNFα and IL-1 which are pro-inflammatory in nature[6].

TLC is a simple and widely used technique for the separation and analysis of compounds. The R_f values of *K. galanga* obtained are the same as the results of Tritripmongkol *et al.*, for ethanol extract - 0.12, 0.32, 0.51, for methanol extract -0.14, 0.30, 0.42, for acetone - 0.10, 0.18, 0.36, for chloroform - 0.12, 0.20, 0.40, 0.46 and petroleum ether extract - 0.63[26]. in GCMS analysis the presence of one of the potent bioactive compounds, Ethyl-p-methoxycinnamate[5], has numerous biological properties such as anti-inflammatory, anti-proliferative, wound healing and anti-angiogenic activity.

Ghasemi *et al.*,[8] observed that the zones of inhibition for Gram-negative bacteria ranged from 9.5mm to 13mm and for Gram-positive bacteria, it was in the range of 10mm to 15mm. In another study, for *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella enterica ser, Typhimurium* and *E. coli*, the zones of inhibition ranged from 3 to 9mm and differential bacterial activity was attributed to the differences in the cell wall of Gram-negative and Gram-positive bacteria[9]. Furthermore, the sample was undergone for the anticancer study against the HeLa Cell Line and the IC_{50} value was found to be 25.04 µg/mL. Vishaka *et al.*, found the IC_{50} value of 44.18 ± 0.65 µL/mL for the HeLa cell line after 24 hours of incubation[27]. This activity denotes the presence of biochemical compounds such as Ethyl-p-methoxycinnamate, which have potent antiproliferative effects. Cells in the early apoptosis stage can be identified by Annexin V and those in the late apoptosis stage can be identified by Propidium Iodide (PI)[23]. Wahyuni *et al.*,[28], reported that *K. galanga* induced apoptotic changes such as changes in nuclear components and cell shape in Ehrlich ascites carcinoma cells[28] with methanolic extract. Similarly, Wang *et al.*,[29] studied the activity of the CL-6 and HuCCT1 cells and which revealed that *K. galanga* extract and Ethyl-p-methoxycinnamate, apoptosis were triggered at IC_{50} and IC_{10} concentrations[29].

5. Conclusion

Prolonged exposure to oxidative stress is a prime cause for the rise in different diseases, inclusive of inflammatory conditions. This study has found that *K. galanga* rhizome has good antioxidant and anti-inflammatory activity. Hence, the use of *K. galanga* rhizome extracts can be proposed to be used as an adjunct antioxidant and anti-inflammatory medication to tide over exacerbations in chronic illnesses. GC-MS showed the presence of many bioactive compounds contributing to the antibacterial, anticancer and apoptotic activity of *K. galanga*, which was further proved using *in vitro* methods in this study. Antibacterial assay showed that the extracts had activity against Gram-positive and Gram-negative bacteria from 12mm to 17mm of inhibition. Anticancer activity against the HeLa cell line showed dose-dependent cytotoxicity and the IC_{50} value was obtained as 25.04 µg/mL. Apoptosis study by flowcytometry showed that the percentage of total apoptotic cells, late and early apoptotic stages were 24.63%, 20.86% and 0.86% respectively. However, *in vivo* clinical studies may be required for its prospective therapeutic application.

6. Acknowledgements

The authors are grateful to the Centre for Bioscience and Nanoscience Research, Coimbatore, for providing laboratory facilities and support.

7. Author Contribution

Jubin conducted research and prepared the initial draft of the manuscript, DR R Ragunathan supervised and identified the research problems, Dr. Jesteena Johney prepared experimental studies and finalized the manuscript, all authors approved the final draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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