



The polyherbal combination Elakanadi Kashayam shows anti-inflammatory, anti-oxidative and anti-cancerous properties

Running Title: Properties of Elakanadi Kashayam

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Abstract

Elakanadi Kashayam is an ayurvedic decoction prescribed for treating various chronic respiratory disorders. It is a highly unexplored medicine and based on the composition of this formulation, it is expected to have properties other than what it is generally prescribed for. We assayed the biochemical properties and evaluated the anti-inflammatory, anti-oxidant and anticancer properties of the Elakanadi Kashayam. We identified the presence of alkaloids, glycosides, phytosterols, flavonoids and tannins along with others in the composition. The in vitro studies proved the anti-inflammatory and anti-oxidant potential along with their ability to deter the proliferation of cancer cells proving their anti-cancer potential too.

Key words: Elakanadi Kashayam, anti-inflammatory, anti-oxidant, anti-cancerous, in vitro assays

Introduction

Ayurveda is an ancient traditional medical system originated in India. It is more personalised medicine with proven scientific evidence of action ^[1]. One such ayurvedic drug Elakanadi Kashayam is commonly prescribed for treating various chronic respiratory disorders like asthma, bronchitis, and allergy. It is a poly herbal combination and the main ingredients in this formulation are *Elettaria cardamomum*, *Piper longum*, *Glycyrrhiza glabra*, *Zingiber officinale*, *Cyperus rotundus*, *Adhatoda vasica*, *Azadirachta indica*, *Tinospora cordifolia*, and *Coleus vettiveroides*. It also contains one part of Dasamuoola, which consists of ten types of roots namely, *Aegle marmelos*, *Premna mucronata*, *Oroxylum indicum*, *Stereospermum suaveolens*, *Gmelina arborea*, *Solanum indicum*, *Solanum xanthocarpum*, *Tribulus terrestris*, *Desmodium gangeticum*, *Uraria picta*. This drug is prepared by boiling one part of each ingredient in 16 parts of water till the volume reduced to 4 parts, then cooled, filtered and bottled as medicine. The medicine is taken at a dose of 5 - 15 ml diluted with 15 - 45 ml of water twice daily before food or as directed by physician.

As, this drug is prescribed for chronic pulmonary diseases, which is an inflammatory condition ^[2], we sought to identify its anti-inflammatory properties. As such most of the major individual ingredients in Elakanadi Kashayam are known to have anti-inflammatory properties and known to target different pathways involved in inflammation involving NF- κ B, COX-2, iNOS, TNF α and IL-1 β ^[3]. But till now there were no studies done to identify the different properties of this herbal composition. As Elakanadi Kashayam is known to have different ingredients that apart from being potent anti-inflammatory, also known to have anti-oxidant and anti-cancerous property, we analysed their potential for all these three properties by different in vitro assays. Also the composition of this drug were evaluated by biochemical assays and by HPTLC technique. Further, the presence of different compounds were confirmed by GC-MS analysis. This is the first time, this drug has been characterised and studied in detail for the in vitro anti-inflammatory, anti-oxidant and anti-cancerous properties and the results have been discussed.

Materials and Methods

Preliminary phytochemical analysis

The dried semi-solid mass of the drug Elakanadi Kashayam is weighed 100 mg and dissolved in 25 ml of water and subjected to preliminary phytochemical analysis using the standard methods^[4]. The presence of alkaloids, quinones, carbohydrates, terpenoids triterpenoids, glycosides, phytosterols, phenolic compounds, steroids, flavonoids, amino acids and tannins were analysed. The presence of different phyto-constituents were detected through prescribed method^[4].

HPTLC profiling of Elakanadi Kashayam

The sample was dissolved in ethyl acetate at 1:1 ratio and chromatographed on aluminium backed HPTLC plates, precoated with silica gel 60F₂₅₄. The sample were dried by passing nitrogen stream and were developed in ascending mode in a twin-trough glass chamber presaturated with 20 ml of mobile phase for 15 min by sealing the chamber air tight with parafilm. The chromatogram was run up to 8 cm from the point of sample application using

n-Hexane: Ethyl acetate: formic acid: acetic acid in different ratios (70: 30: 1.0: 1.0) (v/v/v/v), as the mobile phase. The images were captured at visible and UV light at 366 nm and 254 nm. For qualitative analysis, densitometric scanning was done immediately using Camag TLC scanner 3 with winCATS software (Version 1.4.8).

GC-MS Study

For GC-MS analysis, the Gas chromatography (Agilent: GC: (G3440A) 7890A. MS MS: 7000 Triple Quad GCMS,) equipped with Mass spectrometry detector was used. For the assay, 100 µl of sample was dissolved in 1 ml of suitable solvents and stirred vigorously using vortex stirrer for 10s. The resultant clear extract was used for analysis. The sample was run in GC MS column consisted of DB5 MS (30mm×0.25mm ID ×0.25 µm, composed of 5% phenyl 95% methyl poly siloxane), Electron impact mode at 70 eV; Helium (99.999%) was used as carrier gas at a constant flow of 1ml/min Injector temperature 280°C; Auxilary Temperature: 290°C Ion-source temperature 280 °C. The oven Temperature was programmed from 50°C (isothermal for 1.0 min), with an increase of 40°C/min, to 170°C (isothermal for 4.0 min), then 10°C/min to 310°C (isothermal for 10min) fragments from 45 to 450 Da. Total GC running time is 32.02 min. The compounds are identified by GC-MS Library (NIST & WILEY).

Anti-oxidant Assays

The anti-oxidant potential of the drug was analysed using two different assays, viz., ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging assay and Ferric reducing antioxidant potential (FRAP) assay.

For ABTS assay, the ABTS⁺ radical cation was produced by the reaction of a 7 mM/L ABTS solution with 2.45 mmol/L potassium per sulphate (K₂S₂O₈) and is stored in the dark at room temperature for 12 h before use. The ABTS⁺ solution was diluted with ethanol. Different concentration of test samples viz., 5, 10, 20, 40, 80,160 and 320 µg/ml, standards and Ascorbic acid (5-320 µg/ml) were mixed with the diluted ABTS solution (2.0 ml) and the reaction mixture was allowed to stand at room temperature for 6 min; then, the absorbance was measured using an ultraviolet-visible spectrophotometer at 734 nm. The radical scavenging activity was calculated by the equation:

$$\text{ABTS radical scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where, A₀ is the absorbance of control; A₁ is the absorbance of test.

The FRAP assay was carried out by following the procedure of Benzie and Strain et al., 1999^[5] with minor modification. The FRAP reagent was prepared by mixing 300 mM acetate buffer, 10 ml TPTZ in 40 mM HCl and 20 mM FeCl₃.6H₂O in the proportion of 10:1:1 at 37°C. For each assay, freshly prepared FRAP reagent was mixed with various concentrations (5-320 µg/ml) of test samples, standards and Ascorbic acid thoroughly. An intense blue color complex was formed when ferric tripyridyl triazine (Fe³⁺ TPTZ) complex was reduced to ferrous (Fe²⁺) form and the absorbance at 593 nm was recorded against a reagent blank (3.995 ml FRAP reagent + 5µl distilled water) after 30 min incubation at 37°C.

Anti-inflammatory assay

The anti-inflammatory capacity of the test samples Elakanadi Kashayam was carried out by protein denaturation assay and by Membrane stabilisation assay.

The protein denaturation assay was carried out with minor modification of Altir *et al.* 2021^[6]. The sample was dissolved in DMSO and different concentrations of drug (50, 100, 200, 400, 800 and 1600 µg/ml) was mixed with 1ml of 1mM albumin solution and incubated at 37°C for 15 min. Denaturation was induced by keeping the reaction mixture at 60°C in water bath for 15 min. After cooling, the turbidity was measured at 660 nm. The standard drug Diclofenac sodium at the same concentrations were used as control. Percentage of inhibition of denaturation was calculated by using following formula.

$$\% \text{ Inhibition} = [(OD \text{ of test} - OD \text{ of control}) / OD \text{ of test}] \times 100$$

For Membrane stabilisation assay, fresh whole human blood (5 ml) was used. The packed cells were reconstituted as 40% v/v suspension with isotonic solution (10 mM sodium phosphate buffer). The different concentrations (50 -1600 µg/ml) of Elakanadi Kashayam were mixed with 0.1 ml of 40% RBCs suspension and incubated in water bath at 56 °C for 30 min. As standard, the drug Diclofenac sodium and as negative control, 0.1 ml of RBC mixed with isotonic solution alone was used. At the end of incubation, the tubes were cooled to room temperature and centrifuged at 2500 rpm for 5 min. The absorbance of the supernatant was measured at 560nm and the Percent membrane stabilization activity was calculated by using the following formula.

$$\% \text{ Inhibition of Haemolysis} = (OD \text{ of test} - OD \text{ of control}) / OD \text{ of test} \times 100$$

Anti-cancer activity

The anti-cancer activity of Elakanadi Kashayam was analysed on lung carcinoma cell line-A549 using MTT assay. The cells were plated at the density of 4000 cells per well in 96 well plate and incubated for 24 hours at 37°C in CO₂ incubator. The next day, Elakanadi Kashayam were added at different concentrations ranging from 1, 3, 10, 30, 100, 300, and 1000 µg/ml. The cells were incubated for 48 hours in the CO₂ incubator at 37°C. At 3 hours before the end of incubation period, MTT solution 50 µl/ well from stock of 5 mg/ml was added. The plates were read in Synergy HT microplate reader at 570 nm after dissolving the formazan crystals in DMSO. From the optical densities the percentage growths were calculated using the following formula:

$$\text{Percentage growth} = 100 \times [(T - T_0) / (C - T_0)]$$

Where, T is optical density of test, C is the optical density of control, and T₀ is the optical density at time zero (at the time of compound addition will serve as blank to assess the cytotoxicity). From the percentage growths a dose response curve was generated and IC₅₀ values were calculated.

Statistical analysis

All the results are expressed as mean ± S.E.M. The statistical significance was determined by one-way analysis of variance (ANOVA), followed by a Dunnett's multiple-comparison test with 95% confidence intervals. The P value of less than 0.05 was considered significant.

Results

Biochemical analysis of Elakanadi Kashayam

The analysis of Elakanadi Kashayam by different biochemical methods reveals the presence of alkaloids, carbohydrate, glycosides, phytosterols, flavonoids, amino acids and tannins. But

there were no quinones, terpenoids, triterpenoids, phenolic compounds and steroids identified in these assays (Table 1).

HPTLC analysis

In HPTLC analysis, we observed ten peaks and out of those, two peaks indicates the presence of two major compounds in the sample (Figure 1). A Peak at R_f 0.44 indicated 17.85% of compound A and Peak at R_f-0.66 indicated 61.41 % of compound B. Further analysis is required to identify those two major components

GC-MS Study

The identification of metabolites was accomplished by comparison of retention time and fragmentation pattern with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS. The possible pharmaceutical roles of each bio molecule were identified as per Dr. Duke's Phytochemical and ethnobotanical data base (National Agriculture Library, USA) and others as shown in Table 2. The GC MS profile indicated the presence of some medicinally important molecules such as Cyclobutane-1,1-dicarboxamide, N,N'-di-benzoyloxy-, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, 5,8,11,14-Eicosatetraenoic acid, methyl ester, 7,10,13-Eicosatrienoic acid, methyl ester, Ursodeoxycholic acid, Ethyl iso-allocholate (Figure 2). The roles of each molecule are shown in Table 2 which indicated the role of these molecules in supporting Elakanadi kashayam as a medicine with broad implications.

Antioxidant property of Elakanadi Kashayam

The standard drug Ascorbic acid were used as standard for ABTS and FRAP antioxidant assay. The The IC₅₀ value, that is half maximal inhibitory concentration, was calculated from R² equation obtained from linear thread line from the respective graph of concentration of Elakanadi Kashayam / standard against % inhibition and activity values. The IC₅₀ value for ABTS radical scavenging effect of Elakanadi Kashayam was found to be 273.45 µg/ml and Ascorbic acid was 17.14 µg/ml. Our analysis shows higher radical scavenging effect of Elakanadi Kashayam compared to that of standard at all the analysed concentrations (Figure 3A). The FRAP assay result as indicated in Figure 3B, showed an increase in value with increase in concentration in both test and standard. The results indicate that Elakanadi Kashayam shows comparable FRAP activity as with that of standard ascorbic acid.

Anti-inflammatory assay

The anti-inflammatory property of Elakanadi Kashayam as measured using Protein denaturation assay and Membrane stabilisation assay revealed its potential as anti-inflammatory drug. We observed the highest inhibition of protein denaturation at the highest dose (1600 µg/ml) analysed for both Elakanadi Kashayam and control (Figure 4A). The IC₅₀ value for Protein denaturation activity of Elakanadi Kashayam and standard drug Diclofenac sodium was found to be 420.87 µg/ml and 221.23 µg/ml, respectively. The Elakanadi Kashayam shows better activity by showing less protein denaturation compared to the standard. As for the membrane stabilisation activity, the Elakanadi Kashayam shows a considerable inhibition in the lysis of RBCs but the values are less compared to the control (Figure 4B). The IC₅₀ value for Membrane Stabilisation activity where 795.25 µg/ml and 378.83 µg/ml of Elakanadi Kashayam and Diclofenac sodium, respectively.

Anti-cancer activity

The anti-cancer activity as analysed by the effect of the drug on the proliferation on lung carcinoma cell line A549 reveals a dose depended decrease in proliferation (Figure 5). There is no effect observed till 10 µg/ml and after which we found a gradual decrease in the proliferation of A549 cells with increasing dose of the drug. At 30 µg/ml we found an 18% decrease in growth while the highest dose (1000 µg/ml) analysed showed a great decrease of 78% in proliferation. The IC₅₀ value of this drug was found to be 168 µg/ml.

Discussion

Elakanadi Kashayam is an ayurvedic drug that gains importance during the covid epidemic and was sought as remedy for treating respiratory disorders associated with covid morbidity^[7]. This drug is a poly herbal preparation with the individual herbal components showing proven anti-inflammatory^[3, 8, 9] and/or anti-oxidant^[9-11] and/ or anti-cancerous^[9, 12-14] property. Though the properties of individual herbs are known, this elite combination is never studied for any biochemical and biological properties. Going by the well-known fact that when individual phytochemical components are pooled, there is a synergic effect that is better than the individual components alone^[15], we hypothesised that this combination should have better biological effects. We did an extensive biochemical analysis along with analysing their efficiency as anti-inflammatory, anti-oxidant and anti-cancerous agent.

The initial phytochemical characterisation reveals the presence of alkaloids, carbohydrate, glycosides, phytosterols, flavonoids, amino acids and tannins in this formulation. Alkaloids are the major part of plant secondary metabolites and are associated with anaesthetics, cardio protective, and anti-inflammatory properties^[16]. Glycosides are molecules with attached sugar by glycosidic bonds and are common form of storage chemical in plants. Glycosides also shown to have anti-inflammatory, anti-oxidant and anti-cancerous properties^[17]. Phytosterols are non-nutritive, plant derived sterols with significant pharmacological effects as anti-inflammatory, anti-oxidant, anti-diabetic, antiatherosclerotic agents and chemopreventive agent^[18]. Flavonoids belongs to large family of phenolic compounds and are present in higher abundance in plants. There are various forms of flavonoids available and they are widely researched now owing to their myriad medicinal properties including anti-inflammatory, anti-oxidant, anti-cancer, antibacterial, cardioprotective and immunological properties^[19]. Plant tannins are polyphenolic secondary metabolite compounds and similar to other metabolites, they also show anti-inflammatory, anti-cancer, anti-oxidant properties along with being an antimicrobial, anti-diabetic, wound healing and cardiovascular protection agent^[20].

From the further GC-MS analysis, we identified the presence of medicinally important biomolecules such as Cyclobutane-1, 1-dicarboxamide, N, N'-di-benzoyloxy-, E, E, Z-1, 3, 12-Nonadecatriene-5, 14-diol which have potent anti-oxidant and anti-tumor properties^[21]. The other molecule, Ethyl iso-allocholate has anti-inflammatory property^[22]. The presence of these molecule among the others may contribute to the anti-cancerous, anti-oxidant and anti-inflammatory properties of Elakanadi Kashayam.

To further prove these properties, we carried out various in vitro assay. The anti-oxidant property of Elakanadi Kashayam was analysed using ABTS and FRAP assay. ABTS and FRAP assays are commonly employed method to analyse the in vitro anti-oxidant properties and relies on the ability of the present antioxidants to reduce ABTS^{*+} or Fe³⁺ ions to a colourless or a blue coloured complex respectively^[23]. From the ABTS assay, we identified the IC₅₀ value for radical scavenging effect of Elakanadi Kashayam to be 273.45 µg/ml. The reducing power as analysed by FRAP assay showed a linear correlation to the concentration of drug with an increased activity with increasing concentration of this drug. This results are expected due to the presence of various anti-oxidant molecules in the Elakanadi Kashayam drug formulation.

As this drug is commonly prescribed for chronic pulmonary diseases, which is an inflammatory disease, we decided to analyse the anti-inflammatory property of Elakanadi Kashayam using Protein denaturation and Membrane stabilisation assay. There is a great level of association with the inflammatory diseases and protein denaturation^[24]. So, the substance with the ability to inhibit denaturation of protein, should have potent anti-inflammatory activity. The membrane stabilisation property is linked to their ability to protect the cellular membrane against various insults including inflammatory molecules^[25]. We found that compared to the standard anti-inflammatory drug Diclofenac sodium, the Elakanadi Kashayam is almost two times better as inhibiting protein denaturation and stabilising cell membrane.

Due to the presence of anti-tumor molecules, we also analysed their potential as anti-cancerous drug in the in vitro setting by investigating their effect on the proliferation of lung cancer cell line A549. Our results indicates that at very low concentration of 10 µg/ml, there were no effect but at the higher concentrations, the effect is clearly visible with a maximum decrease in proliferation of up to 78% was observed at 1000 µg/ml concentration.

From the in vitro analysis for anti-oxidant, anti-inflammatory and anti-cancerous of this drug, our results clearly indicates that this Ayurveda drug Elakanadi Kashayam have all these properties. It is really interesting that with the variety of ingredients, it is a drug with multiple properties and could have wider clinical application. This drug can be prescribed for any of these indication after in vivo confirmatory studies.

Summary

Elakanadi Kashayam is a poly herbal formulation with lots of unproven medicinal properties. Our study proved their anti-inflammatory, anti-oxidant and anti-cancerous properties by in vitro studies. Further in vivo studies needs to be done to prove these effects and to harness the full potential of this drug.

Authors' contributions

SK: Design of studies, data collection, analysis and manuscript writing

LS: Design of studies, Acquisition of data, analysis and interpretation, final approval of the manuscript

PK: Design of studies, data collection and analysis

MR: Design of studies, data analysis and interpretation

PA: Design of studies, data analysis and interpretation

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Declarations regarding ethical issues: No humans or animals are used in this study

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Table 1. Phytochemical analysis of Elakanadi Kashayam

S. No	Phytochemical list	Presence (+) or absence (-)
1.	Alkaloids	+
2.	Quinones	-
3.	Carbohydrate	+
4.	Terpenoids	-
5.	Triterpenoids	-
6.	Glycosides	+
7.	Phytosterols	+
8.	Phenolic compounds	-
9.	Steroids	-
10.	Flavonoids	+
11.	Aminoacids	+
12.	Tannins	+

Table 2. GC MS profile of Elakanadi Kashayam showing the retentions values, types of possible compound, their molecular formulae, molecular mass, peak area and their medicinal roles.

Ret. Time	Compound	Mol. Formula	Mol. Mass	% Peak Area	Possible medicinal role
5.76	Cyclobutane-1,1-dicarboxamide, N,N'-di-benzoyloxy-	C ₂₀ H ₁₈ N ₂ O ₆	382.1	48.65	Anaphylactic, Antitumor, Arylamine-N-Acetyltransferase-Inhibitor, Decreases Norepinephrine Production, Down regulates nuclear and cytosol androgen reuptake, GABA-nergic,

					Increases natural killer cell activity, Inhibits Production of Tumor Necrosis Factor, Myo-neuro-stimulant, N-Cholinolytic, NADH-Oxidase-Inhibitor, NADH-Ubiquinone-Oxidoreductase-Inhibitor
20.39	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	C19H34O2	294.3	1.46	Antitumor, antimicrobial, antidote, Cytochrome-P450-2E1-Inhibitor, Decreases C-Teleopeptide Excretion, Decreases Deoxypyridinoline Excretion, Decreases Endothelial Leukocyte Adhesion, Decreases Epinephrine Production, Decreases Oxalate Excretion
24.21	5,8,11,14-Eicosatetraenoic acid, methyl ester	C21H26O2	310.2	4.28	Acidifier, Arachidonic acid Inhibitor, Increases Aromatic Amino acid decarboxylase activity, Inhibits production of uric acid, Urine acidifier, 17 beta hydroxysteroid dehydrogenase inhibitor, Catechol o methyl Transferase inhibitor, methyl donar, methyl guanidine inhibitor
25.75	7,10,13-Eicosatrienoic acid, methyl ester	C21H36O2	320.3	5.55	Acidifier, Arachidonic acid Inhibitor, Increases Aromatic Amino acid decarboxylase activity, Inhibits production of uric acid, Urine acidifier, 17 beta hydroxysteroid dehydrogenase inhibitor, Catechol o methyl Transferase inhibitor, methyl donar, methyl guanidine inhibitor
25.78	Ursodeoxycholic acid	C24H40O4	392.3	2.71	Acidifier, arachidonic acid inhibitor, increases aromatic amino acid decarboxylase activity, inhibits production of uric acid
26.10	Ethyl iso-allocholate	C26H44O5	436.3	0.99	Anti-coagulant, anti-dyspeptic, anti-inflammatory, mucolytic, proteo-lytic
26.95	Dasycarpidan-1-methanol, acetate (ester)	C20H26N2O2	326.2	3.78	Not known
27.09	2,2-Bis(3-hydroxy-5-methylbenzyl)-7-methylindan-1-one	C26H26O3	386.2	2.09	17-beta-hydroxysteroid dehydrogenase inhibitor, Aryl hydrocarbon hydroxylase inhibitor, testosterone hydroxylase inducer
27.29	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	C35H68O5	568.5	6.58	Acidifier, Arachidonic acid Inhibitor, Increases Aromatic Amino acid decarboxylase activity, Inhibits production of uric acid, Urine acidifier
28.47	4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H-indol-2-yl]-.alpha.-methyl-, methyl ester	C23H32N2O4	400.2	1.89	Not known

28.81	Lycoxanthin	C40H56O	552.4	1.00	Not known
29.49	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	C57H104O6	884.8	19.27	Not known

Figure 1. HPTLC profile of Elakanadi Kashayam. A, B- TLC plate photographed under UV at 245 nm and 366 nm. C, D- HPTLC chromatogram collected at 245 nm and 366 nm

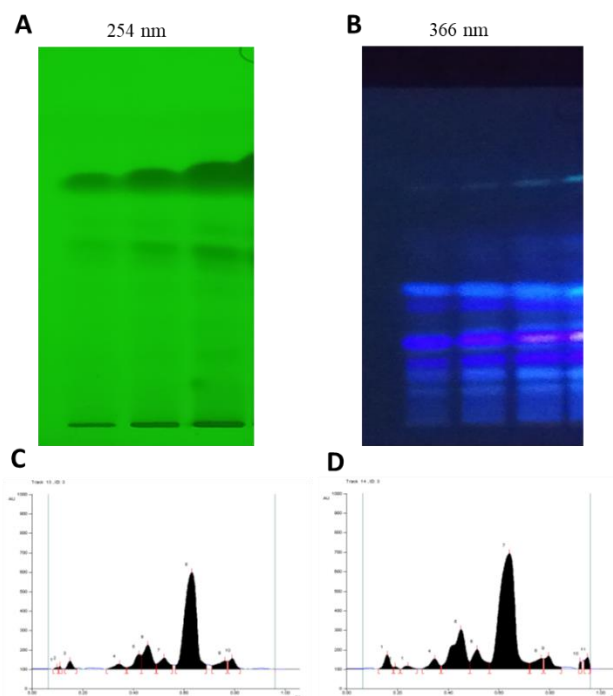


Figure 2. GC MS chromatogram of Elakanadi Kashayam showing peaks

Qualitative Compound Report

Data File	220620020.D	Sample Name	Elakanadi Kashayam
Sample Type		Position	116
Acq Method	GC Screening Method.M	Acquired Time	24-06-2020 PM08:41:03
Comment			

User Chromatogram

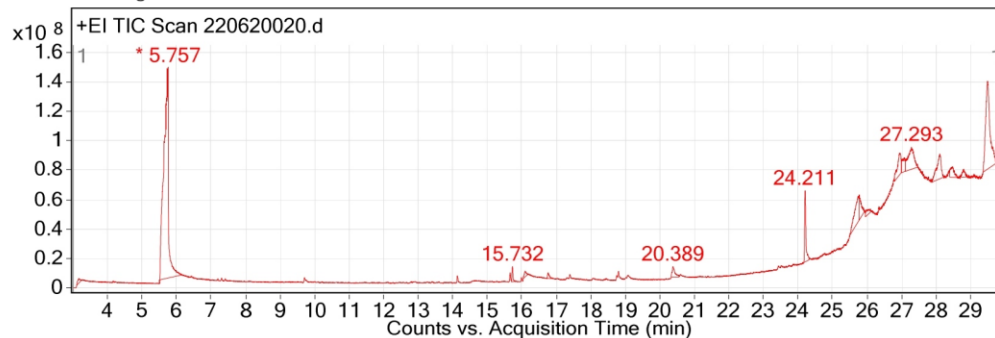


Figure 3: Antioxidant activity of radical scavenging activity of Elakanadi Kashayam compared to Ascorbic acid by A- ABTS and B- FRAP assay

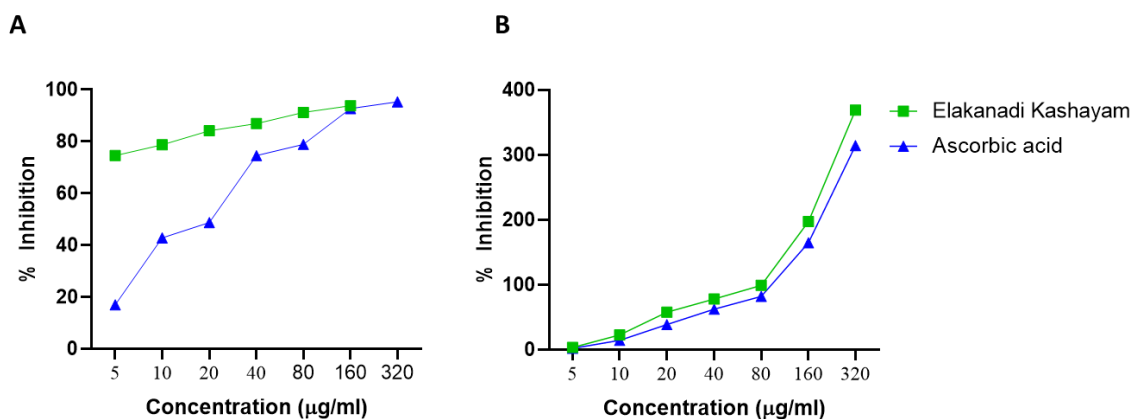


Figure 4: Anti-inflammatory property of Elakanadi Kashayam compared to Diclofenac sodium as measured using A- Protein denaturation assay and B- Membrane stabilisation assay

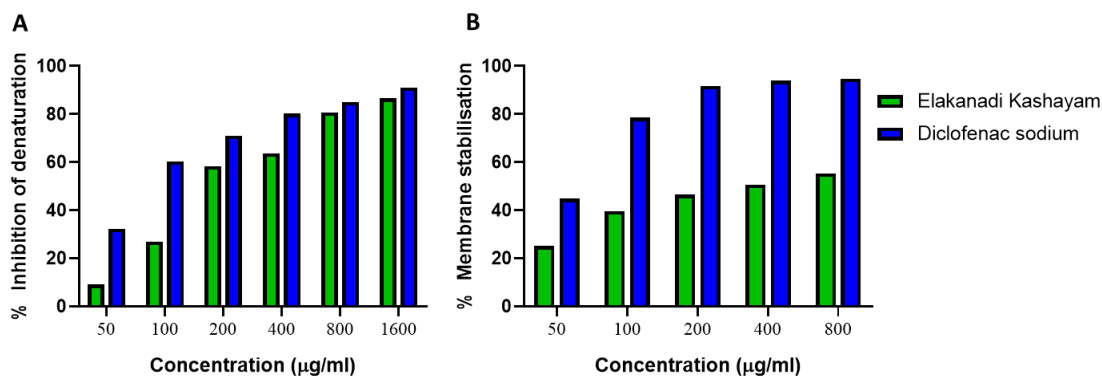


Figure 5: Anti-cancer effect of Elakanadi Kashayam on lung carcinoma cell line A549 by MTT assay

