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

Running Title: Properties of ElakanadiKashayam

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Abstract

ElakanadiKashayam 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 ElakanadiKashayam. 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: ElakanadiKashayam, 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 suchayurvedic drug ElakanadiKashayam 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*Elettariacardamomum, Piper longum***,** *Glycyrhizaglabra***,** *Zingiberofficinale***,** *Cyperusrotundus***,** *Adhatodavasica***,** *Azadirachtaindica***,** *Tinosporacordifolia***,** and *Coleus vettiveroides*. It also contains one part of Dasamuoola, which consists of ten types of roots namely,*Aegle marmelos, Premnamucronata, Oroxylumindicum, Stereospermumsuaveolens, Gmelinaarborea, Solanum indicum, Solanum xanthocarpum, Tribulusterrestris, Desmodiumgangeticum, Urariapicta*.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 suchmost of the major individual ingredients in ElakanadiKashayam are known to haveanti-inflammatory properties and known to target different pathways involved in inflammation involving NF-κB, COX-2, iNOS, TNFα and IL-1β[3].But till now there were no studies done to identify the different properties of this herbal composition. As ElakanadiKashayam 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 vitroanti-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 drugElakanadiKashayam is weighed 100 mganddissolved 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 weredetected through prescribed method[4].

HPTLC profiling of ElakanadiKashayam

The sample was dissolved in ethyl acetate at 1:1 ratio and chromatographed on aluminium backed HPTLC plates, precoated with silica gel $60F_{254}$. The sample were dried by passing nitrogen stream and were developed in ascending mode in a twin-trough glass chamberpresaturated 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, theGas chromatography (Agilent: GC: (G3440A) 7890A. MSMS: 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 ($K_2S_2O_8$) 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 (5-320μg/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-visiblespectrophotometer at 734 nm. The radical scavenging activity was calculated by the equation:

$$\text{ABTS radical scavenging effect (\%)} = [(A0 - A1)/A0] \times 100$$

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 mMHCl and 20 mM $FeCl_3.6H_2O$ 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 andAscorbic acid thoroughly. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+} TPTZ) complex was reduced to ferrous (Fe^{2+}) 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 ElakanadiKashayam 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 of1mM 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. Aftercooling, the turbidity was measured at 660 nm. The standard drug Diclofenac sodiumat the same concentrations were used as control. Percentage of inhibition of denaturation was calculated by using following formula.

% Inhibition = [(OD of test - OD of control)/OD of test] x 100

For Membrane stabilisation assay,fresh whole human blood (5 ml) was used. The packed cells were reconstitutedas 40% v/v suspension with isotonic solution (10 mM sodium phosphate buffer). The different concentrations (50 -1600 µg/ml) of ElakanadiKashayam 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. Atthe end of incubation, the tubes were cooled to room temperature and centrifuged at 2500 rpm for 5 min. Theabsorbance of the supernatant was measured at 560nm and the Percentmembrane stabilization activity was calculated by using thefollowing formula.

% Inhibition of Haemolysis =(OD of test - OD of control) /OD of test x 100

Anti-cancer activity

The anti-cancer activity of ElakanadiKashayam 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 CO_2 incubator. The next day, ElakanadiKashayam were added at different concentrations ranging from 1, 3, 10, 30, 100, 300, and 1000µg/ml. The cells were incubated for 48 hours in the CO_2 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:

Percentage growth= 100× [(T-T0)/(C-T0)]

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 IC_{50} 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 ElakanadiKashayam

The analysis of ElakanadiKashayam 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 steroidsidentified 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 Rf 0.44 indicated 17.85% of compound A and Peak at Rf-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-Eicosatetraynoic 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 Elakanadikashayam as a medicine with broad implications.

Antioxidant property of ElakanadiKashayam

The standard drug Ascorbic acid were used as standard for ABTS and FRAP antioxidant assay. TheThe IC50 value, that is half maximal inhibitory concentration, was calculated from R^2 equation obtained from linear thread line from the respective graph of concentration of ElakanadiKashayam / standard against % inhibition and activity values. The IC_{50} value for ABTS radical scavenging effect of ElakanadiKashayam was found to be 273.45 µg/ml and Ascorbic acid was 17.14µg/ml.Our analysisshows higherradical scavenging effectof ElakanadiKashayamcompared 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 ElakanadiKashayam shows comparable FRAP activity as with that of standard ascorbic acid.

Anti-inflammatory assay

The anti-inflammatory property of ElakanadiKashayam 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 ElakanadiKashayam and control (Figure 4A). The IC_{50} value forProtein denaturation activity of ElakanadiKashayamand standard drug Diclofenac sodium was found to be 420.87 µg/ml and 221.23 µg/ml, respectively. The ElakanadiKashayam shows better activity by showing less protein denaturation compared to the standard. As for the membrane stabilisation activity, the ElakanadiKashayam shows a considerable inhibition in the lysis of RBCs but the values are less compared to the control(Figure 4B). The IC_{50} value for Membrane Stabilisation activity where 795.25 µg/ml and 378.83 µg/ml of ElakanadiKashayam 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/mlwe found an 18% decrease in growth while the highest dose (1000 μg/ml) analysed showed a great decrease of 78% in proliferation. The IC_{50} value of this drug was found to be 168 μg/ml.

Discussion

ElakanadiKashayam is an ayurvedic drugthat 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 chemopreventiveagent [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 ElakanadiKashayam.

To further prove these properties, we carried out various in vitro assay. The anti-oxidant property ofElakanadiKashayam 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 Fe^{3+} ions to a colourless or a blue coloured complex respectively[23]. From the ABTS assay, we identified the IC_{50} value for radical scavenging effect of ElakanadiKashayamto 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 ElakanadiKashayam 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 ElakanadiKashayam 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 ElakanadiKashayam 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 ElakanadiKashayam 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

ElakanadiKashayam 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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Declaration: All the authors declare that the manuscript has been read and approved by all the authors, and the requirements for authorship as stated earlier in this document have been met, and each author believes that the manuscript represents honest work.

Declarationsregarding preprints: All the authors declare that if a manuscript accepted, we assure that the DOI of the manuscript published in the preprint repository will be linked to the DOI of the paper published in Current Science.

Declarations regarding ethical issues: Nohumans or animals are used in this study

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Table 1. Phytochemical analysis of ElakanadiKashayam

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

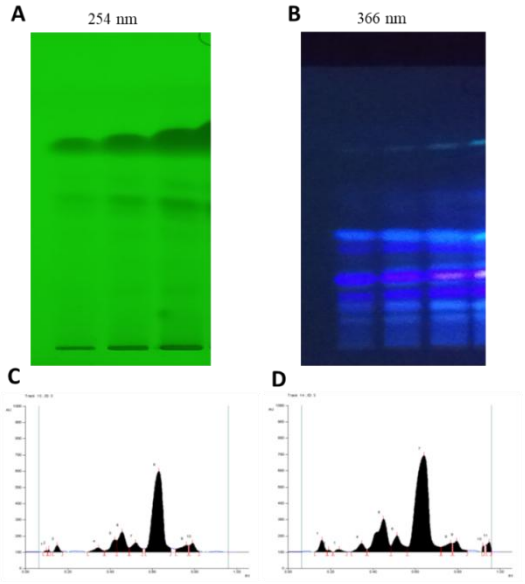
Table 2. GC MS profile of ElakanadiKashayamshowing the retentions values, types of possible compound, their molecular formulae, molecular mass, peak area and their medicinal roles.

Ret. Time	Compound	Mol. Formula	Mol. Mass	% Peak Area	Possible medicinal role
5.76	Cyclobutane-1,1-dicarboxamide, N,N'-di-benzoyloxy-	C20H18N2O6	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 Endothilial Leukocyte Adhesion, Decreases Epinephrine Production, Decreases Oxalate Excretion
24.21	5,8,11,14-Eicosatetraynoic 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-Piperidineaceticacid,1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H- indol-2-yl]-.alpha.-methyl-, methylester	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 ofElakanadiKashayam. A, B- TLC plate photographed under UV at 245 nm and 366 nm. C, D- HPTLC chromatogram collected at 245 nm and 366 nm



2w3e

Figure 2. GC MS chromatogram of ElakanadiKashayam showing peaks

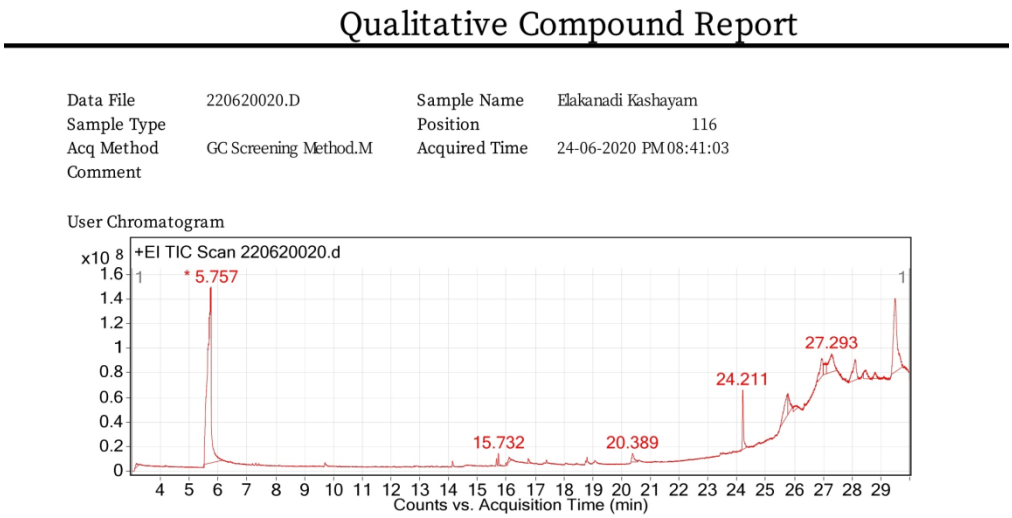


Figure 3: Antioxidant activity of radical scavenging activity of ElakanadiKashayamcompared to Ascorbic acid by A-ABTS and B- FRAP assay

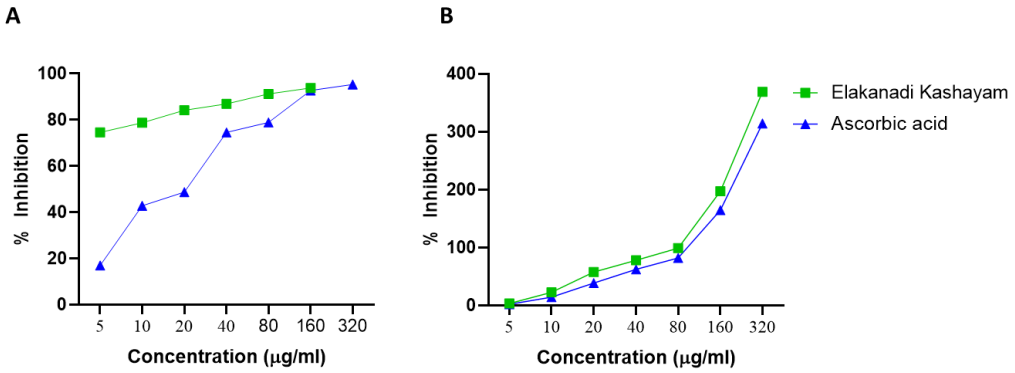


Figure 4: Anti-inflammatory property of ElakanadiKashayam compared to Diclofenac sodium as measured using A- Protein denaturation assay and B- Membrane stabilisation assay

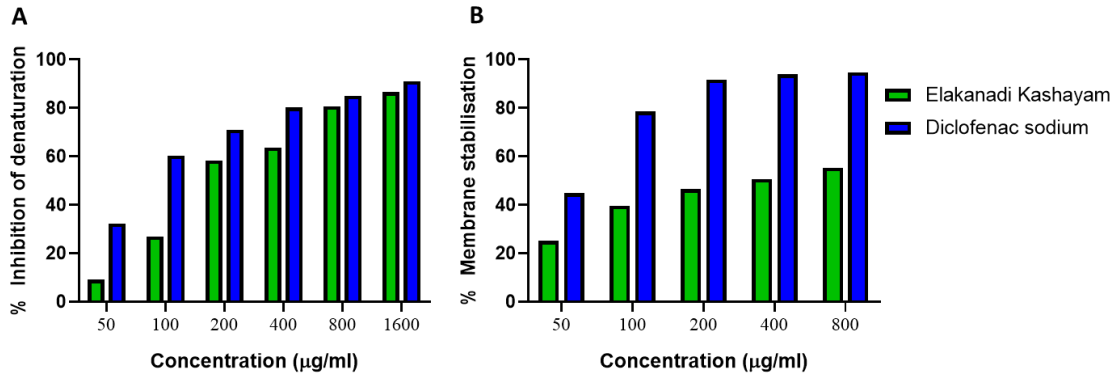


Figure 5: Anti-cancer effect of ElakanadiKashayam on lung carcinoma cell line A549 by MTT assay