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Characterization of essential oil from *Cinnamomumtamala* Bark and *Trachyspermum ammi* Seed, its biological functions assessment

Dr.Krishnaveni M, Tamilselvan M, Praveen R

Department of Biochemistry, Periyar University, Salem-636 011.

Corresponding author

Dr. M. Krishnaveni M.Sc., M.Phil., Ph.D., MBA., BGL., M.A CCJA

Assistant Professor, Department of Biochemistry,

Periyar University, Salem 636 011

Mobile : 9894829823, E.Mail: logasarvesh@gmail.com

Abstract

Essential oil as concentrated hydrophobic liquid comprising of volatile chemical compounds from aromatic plants and could be extracted from different parts. Since, essential oil possess several pharmacological properties it was decided to extract essential oil from commonly available food source from *Cinnamomum tamala* bark and *Trachyspermum ammi* seed. The yield obtained was found to be 2.4% on extraction using clevenger apparatus during the period of 2-3 hrs. The extracted essential oil was assessed via FTIR which showed the presence of functional groups.While,GC-MS showed highest peak area percent of 80.65-Cinnamaldehyde for *Cinnamomum tamala* bark essential oil. While, highest peak area percent was observed with Phenol-79.51 for *Trachyspermumammi* seed. Total phenolics, flavonoid content was0.29±0.02mg/g, 0.19±0.03mg/g and the observed Phosphomolybdenum, Reducing power, Metal chelating activity of *Trachyspermum ammi* seed essential oil was found to be 0.33±0.03mg/g, 0.37±0.03mg/g,0.15±0.02mg/grespectively. While nitric oxide scavenging activity was high 0.48±0.02mg/g with *Cinnamomum tamala* bark essential oil. The antibacterial activity was good with essential oil extracted from *Cinnamomum tamala* bark and *Trachyspermum ammi* seed when tested against *Escherichia coli* MTCC 1692, *Klebsiella pneumonia* MTCC 7403, *Pseudomonas aeruginosa* MTCC 2581, *Staphylococcus aureus* MTCC 7443, *Salmonella enteric* MTCC 8587.All the bacteria studied *Escherichia coli* MTCC 1692, *Klebsiella pneumonia* MTCC 7403, *Pseudomonas aeruginosa* MTCC 2581, *Staphylococcus aureus* MTCC 7443, *Salmonella enteric* MTCC 8587 showed good zone of inhibition. Results of anticancer activity studied by MTT assay showed IC50 value of 32.24µg/ml for essential oil from *Cinnamomum tamala* bark. While, for *Trachyspermum ammi* seed it was 112.5µg/ml.The morphological changes such as reduced cell number and shrinkage of HSC-3 cells were noticed in MTT assay.

Key words: Antibacterial, Anticancer,Antioxidant,Essential oil,FT IR,GC-MS, *Cinnamomum tamala* bark and *Trachyspermum ammi* seed.

1.Introduction

The use of essential oils was first documented in traditional Chinese, Indian medicine between 2000 to 3000 BCand in Greek history between 400 and 500 BC.(Baser,et.al., 2010 and Pauli, et.al., 2009) Theterm essential oil was defined by Paracelsus von Hohenheim during 16th century. (Pichersky,et.al., 2006) Essential oils are hydrophobic, concentrated colorless liquid comprising of volatile chemical constituents such as terpenes, hydrocarbons oxygenated derivatives such as aldehydes, ketones, epoxides, alcohols, and esters(Rehman, et.al., 2007) synthesized by secondary metabolism of aromatic plants and its parts likeseed, bark, peel of fruits, leaves, flowers, oil glands, cuticles etc, soluble in alcohol, ether, with characteristic odor and molecular weight below 300. (Mahatok,et.al., 2019) Several methods like fermentation, effleurage, extraction, expression, steam distillationwere adopted for the extraction. But, the best method was steam distillation process as it yields fragrant mix of compounds as pristine oil for commercialization,considering it to be pure and free from impurities (Burt, 2004 and Tajkarimi, et.al.,) Thecomposition not only depend on the geographical location but also on the maturity of the plant parts used for the extraction. (Benabdelkader, et.al 2011 and Kiran, et.al., 2013)The mechanism of action of essential oil depend on the presence of one or more functional groups in them.(Dorman, et.al., 2000)They are used since olden day for its medicinal, health benefits as it possessing antibacterial, antiviral, analgesic, anti-inflammatory, anticancer, antiprotozoal, detoxifying, gastro-protective, antioxidant (Sun, et.al., 2017) properties and also in aroma therapy to treat various diseases related to respiratory tract,gynecological, andrological, cardiovascular, nervous system, skin infections, and also in food preservation because essential oils are safeto use. Their ability to fight against bacteria was more towards gram positive bacteria when comparedto gram negative bacteria. (Raut,et.al., 2014 and Prakash, et.al., 2005) World Health Organization survey 1993 states, that patients in India (80%), Burma (85%), Bangladesh (90%) were treated using traditional medicines.(Smith, et.al., 1998) Traditional medicines were used in the treatment of dental diseases too as it affect the general health mainly, which plays a major role in maintaining the quality of life, working capacity of a person.(Palombo, et.al., 2011) Since, essential oils possess enormous health benefits,we would like to extract essential oil from *Cinnamomum tamala* bark, *Trachyspermum ammi* seed, and analysed the phyto-constituents present via, FTIR, GCMS and its biochemical properties like carbohydrate, protein, antioxidant, antibacterial activity was tested against *Escherichia coli* MTCC 1692, *Klebsiella pneumonia* MTCC 7403, *Pseudomonas aeruginosa*MTCC 2581, *Staphylococcus aureus*MTCC 7443, *Salmonella enteric* MTCC 8587and also its anticancer activity against human oral squamous carcinoma cell line HSC-3.

2.MATERIALS AND METHODS

2.1 Sample Collection

The dry *Cinnamomumtamala*bark,*Trachyspermumammi*seedswere purchased from a shop at Salem, Tamil Nadu, India. The samples werefreed from dust, debris,stones.

2.2 Essential Oil Extraction

100gm dried *Cinnamomumtamala*bark, *Trachyspermumammi*seedswere subjected to hydro-distillation process usingClevenger's apparatus at 100°Cfor 4hrs.The obtained essential oil was dried over anhydrous sodium sulphate and stored in a vial away from light at 4°C. (Fadil, et.al., 2015) The extracted essential oil was dissolved in 90% ethanol in the ratio of 1:1 and the diluted essential oil was used for further biological analysis. The essential oil yield obtained was 2.4% estimated on a dry weight basis and the yield of essential oil was calculated by formulae:Yield (%) = Amount of extracted oil / Amount used (g) x 100.

2.3 Fourier transform infrared analysis

Potassium bromide pellet method was used for the FTIR analysis. (Frank., 1997)The pellet was prepared by mixing 0.1% essential oil with 200 to 250mg of potassium bromide powder,finely pulverized, pellet was obtained. The spectrum was recorded using Bruker Tensor 27 spectrometer, wavelength range(400-4000cm^{-1}), resolution (4cm^{-1}), scanning speed (2mm/sec).

2.4 Gas Chromatography-Mass Spectrophotometry

Chromatogram of GC-MS was obtained using Scion 436-GC Bruker-Triple quadruple mass spectrophotometer (IIFPT,Thanjavur) with fused silica capillary column BR-5MS (5% Diphenyl95% Dimethyl poly siloxane),30mx0.25mmID x 0.25m df. The program set for column oven temperature was 80°C hold for 2 min, Up to 160°C at the rate of 20°C/min-No hold, Up to 280°C at the rate of 5°C/min-No hold, Up to 300°C at the rate of 20°C/min-10 min hold, Injector temperature 280°C, total running time was 36min. The inlet source temperature (280°C) and source temperature was set at 250°C, ionization mode, ionization at 70-eV ionization energy. For single scan analysis, the scan range was set from m/z 40 to 600; Solvent Delay: 0-3.5 min; the injection volume was 2µl. (Franelyne, et.al., 2016)

2.5 Determination of Total phenol

Folin-ciocalteau method was used for total phenol analysis.(Kaur , et.al., 2002) To 0.1ml essential oil added Folinciocalteau reagent (5 ml, 1:10 dilution) set aside for 5 min and added aqueous $NaCo_3$ (4ml, 1M). Kept at room temperature for 15min. read at 765nm.The reference compound used was phenol and expressed in terms of Gallic acid equivalent (mg/g).

2.6 Estimation of flavonoids

Aluminium chloride method was adopted for the analysis of total flavonoid. (Chang, et.al., 2002) To 0.1ml essential oil added $AlCl_3$ (0.1ml,10%) mixed wellandincubated for 30min., measured the absorbance at 415nm. Quercetin was used as astandardand expressed the results as mg quercetin equivalent/g.

2.7 Nitric oxide scavenging activity

Sodium nitroprusside (10mM) in phosphate buffered saline was mixed with 0.1ml essential oil and incubated at room temperature for 150min. After incubation added 0.5ml Griess reagent and measured the absorbance at 546nm.Quercetin was used as a positive control.(Lee, 1992),(Chakraborthy, 2009)

2.8 Reducing power assay

To 0.1ml essential oil,added phosphate buffer (2.5ml, 0.2M, P^H 6.6), potassium ferriccyanide (2.5ml, 1%), incubated at 50°c for 20min. and the reaction was stopped by 1.0 ml trichloro acetic acid (10%), centrifuged for 10min. at 3000rpm.To 1.5ml supernatant, added 1.5ml distilled water, $FeCl_3$ (0.1ml, 0.1%), mixed the content well, after 10min. incubationmeasured the absorbance at 700nm. Vitamin C was used as a reference compound. (Oyaizu, 1986)

2.9 Total antioxidant activity

Phospho-molybdenum method was used to reduce Mo (V1) to Mo (V) by the essential oil and later green phosphate/Mo (V) complex formation at acidic pH.(Prieto, 1999)

2.10 Metal chelating activity

To 0.1ml essential oil added 0.05ml 2mM $FeCl_2$. The reaction was initiated with 160µl(5mM) Ferrozineaddition and kept at room temperature for 10min., measured the absorbance at 562nm. (Dinis,et.al., 1994)

2.11 Estimation of carbohydrate

The total carbohydrate was analysed by Anthrone method. (Hedge, 1962)To0.1ml essential oil added 4ml anthrone reagent, heated in a boiling water bath for 8 minutes. The tubes were cooled and read at 630nm. The standards were developed with glucose. Standard graph plotted was used to find out concentration of glucose present in the unknown/ sample.

2.12 Estimation of protein

The total protein was estimated by Lowry's method. (Lowry, et.al., 1951) To 0.1ml essential oil added 2ml alkaline copper reagent, mixed well, incubated for 10min., added 0.2ml Folinciocalteau reagent (diluted in the ratio of 1: 2) and kept at room temperature for 30min., read at 660nm. The standards were developed with bovine serum albumin.

2.13 Estimation of aminoacid

The amino acid was assessed by Ninhydrin method. (Yemm, et.al 1955)To 0.1 ml essential oil added 1ml ninhydrin solution dissolved in Butanol: Acetone.With mild stirring, the contents were heated for 4-7 min. at 80-100ºC, cooledand read at 570nm.. Tyrosine was used for developing standards.

For all estimations readings were taken using UV spectrophotometer Schimadzu Model 1800. Standard graph was plotted for all experiments using their respective standards and the samples were plotted against the standard by taking concentration in X axis and OD in Y axis. Each experimentswere performed six times. The Mean and Standard deviation (S) was calculated by using the following formula: Mean = Sum of x values / n (Number of values), $s = \frac{\sqrt{\sum(X-M)^2}}{n-1}$

2.14 ANTIBACTERIAL ACTIVITY- Disc Diffusion Assay

Cinnamomum tamala bark, *Trachyspermum ammi* seed essential oil were tested against *Escherichia coli* MTCC 1692, *Klebsiella pneumonia* MTCC 7403, *Pseudomonas aeruginosa* MTCC 2581, *Staphylococcus aureus* MTCC 7443, *Salmonella enteric* MTCC 8587.Disc diffusion method was adopted for antibiotic susceptibility test. Mueller hinton agar plates were prepared by pouring 15ml molten media into sterile petriplates, solidified for 5min. and then 0.1% inoculum suspension was swabbed uniformly and dried for 5min. In the Kirby-Bauer test, small filter disks soaked with essential oil 5µl,10µl,15µl and Chloramphenicol 30µg/disc (standard antibiotic) was kept on the surface of the agar allowed to diffuse for 5min.,incubated at 37°C for 24h in order to diffuse into the surrounding agar for zone of inhibition formation. The inhibition zones formed were measured using transparent scale in millimeter. (Bauer, 1966)

2.15 Anticancer activity

The extracted essential oil from *Cinnamomum tamala* bark, *Trachyspermum ammi* seed was tested for in vitro cytotoxicity against HSC-3 cells by MTT assay.(Mosmann, 1983) The cultured HSC-3 cells were harvested by trypsinization, pooled in a 15ml tube. Then, the cells were plated at a density of 1×105 cells/ml/well into the 96 well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24-48h at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the extracted essential oil in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24h. After the incubation period, MTT (20 µL, 5mg/ml) was added into each well and the cells were incubated for another 2-4h until purple precipitates were clearly visible under an inverted microscope. The wells were aspirated and then washed with 1X PBS. Later, the formazan crystals formed were dissolved in DMSO and the absorbance was measured at 570nm using a micro plate reader (Thermo Fisher Scientific, USA) and the cell viability (%) and IC50 value was calculated using Graph Pad Prism 6.0 software (USA).

3. Results and Discussion

3.1 FTIR Spectrum of *Cinnamomumtamala bark*and *Trachyspermumammi* seed essential oil

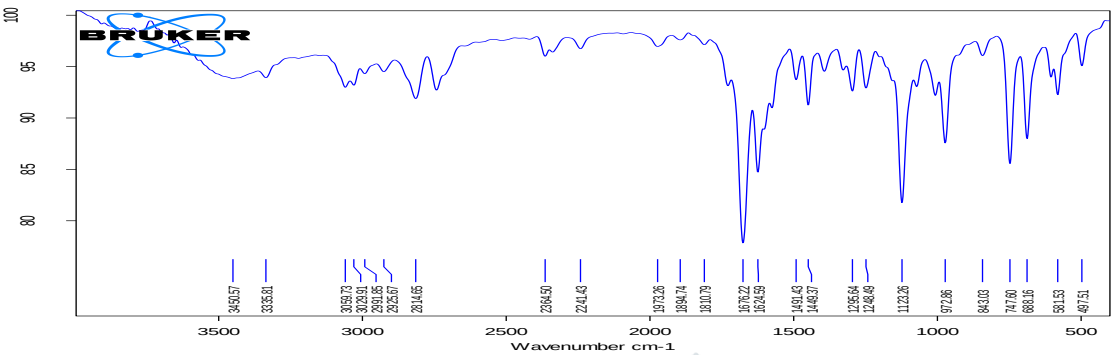


Fig.1 Showing FTIR Spectrum of *Cinnamomumtamala*barkessential oil

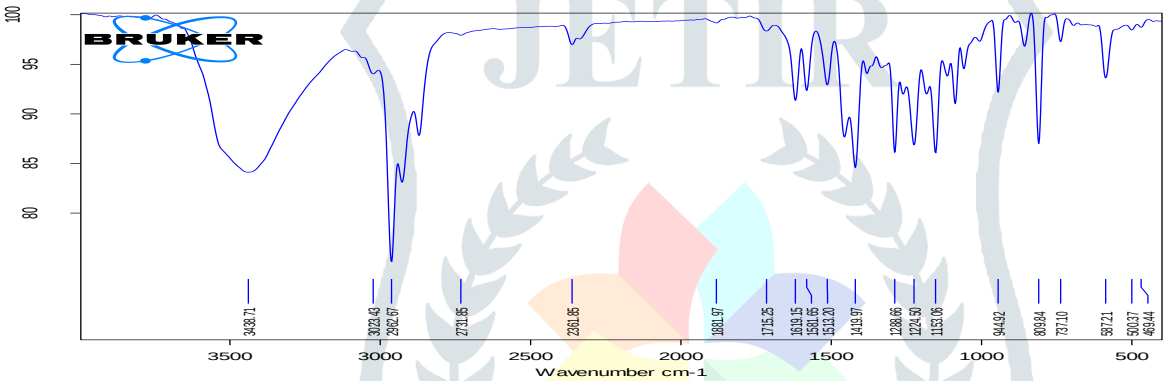


Fig.2 Showing FTIR Spectrum of *Trachyspermumammi*seed essential oil

Figure.1 and 2demonstrates the results of Fourier transform infrared analysis. This analytical technique is widely used in research as sample preparation is easier and much faster in analysis. The stability of chemical constituents, functional groups of the active components depending on the peaks found through stretching and bending vibrations in the region of infrared radiation were assessed by FTIR. Infrared absorption below 1000cm-1 corresponds to C-H bending vibrations, the absorptions from 997 to 1130cm-1 represents stretching vibrations of C-O of monosaccharides, oligosaccharides, while the absorption at 1150- 1270cm-1 relates to stretching vibrations of carbonyl C-O, or O-H bending. Whereas, absorptions at region 1300 – 1450cm-1 signifystretching vibrations of C-O amide, C-C stretching from the phenyl groups and the absorption within 1500-1600cm-1 region represent aromatic domain, N-H bending vibration. The absorptions between 1600-1760cm-1 embodies the bending vibrations of N-H amino acids, C=Ostretching –aldehyde, ketone, esters. The absorptions within 2800-2900 cm-1 attributes to C-H stretching specific to CH_3, CH_2 from lipids and methoxy derivatives and C –H aldehyde as well as from cisdouble bonds. While, absorption at 3350 signifies vibrations of the OH group from water, alcohols, phenols, amides, aldehyde, ketone, esters.

3.2 GC-MS of *Cinnamomum tamala* bark and *Trachyspermum ammi* seed essential oil

Table.1 Showing Components identified in *Cinnamomum tamala* bark essential oil by GC-MS

S.No	RT (min)	Name of the compound	Peak Area %
		Paatai	
1	4.85	Benzenepropanal	0.33
2	5.87	(Z)-3-Phenylacrylaldehyde	0.84
3	6.88	Cinnamaldehyde, (E)-	80.65
4	7.35	2-Propen-1-ol, 3-phenyl-	1.94
5	8.49	Copaene	0.22
6	9.43	Acetic acid, cinnamyl ester	10.93
7	10.26	à-Muurolene	0.31
8	10.42	Cinnamaldehydediethylacetal	0.57
9	10.64	(Z)-2-Methoxycinnamaldehyde	3.69
10	12.10	.tau.-Muurolol	0.54

The cinnamaldehyde a flavonoid identified with 80.65 as a peak area percent possesses antileukaemic,(Adfa, et.al 2016)antimicrobial,(Uthairatsamee, et.al., 2008),(Farah, et.al., 2013)anti-inflammatory, (Khan, 2014), (Hong, et.al., 2002), (Pukdeekumjorn, et al.,2016), antioxidant,(Pardede, et al., 2017) antityrosinase,cytotoxic, (Farah, et.al., 2013), Analgesic.(Mustaffa, et. al., 2010)haemagglutinating, (Li, et. al., 1996)anthelmintic activity,(Gogoi,et al., 2014)hepatoprotective,(Pardede, et al., 2017) Antidiabetic, (Jia,et. al., 2009) RNA N-glycosidase activity, (Li, et. al., 1996)antiviral, (Silprasit, et.al., 2011) anticancer,(Ghalib,et. al., 2012), (Lin, et.al., 2007) antioxidant (Prasad, et.al., 2009) activity. The acetic acid, cinnamyl ester identified with peak area percent 10.93 also called as cinnamylacetatewas used in perfume preparation in order to add fragrance, and also as modifiers in nuts and berry. Cinnamyl alcohol, used in photosensitive polymer production, developing ink for multicolor printing, animal repellent, insect attractant synthesis and also as a fragrance compound. (Charlene, et.al., 2005)2 methoxycinnamaldehyde obtained with peak area percent of 3.64 induces amelioration of ischemia, reperfusion,(Hwa, et.al., 2012) reduces atherosclerosis, inhibits cell proliferation in human aortic smooth muscle cells. (Young-Heem, et.al., 2017)

Table. 2 Showing Components identified in *Trachyspermumammi* seed essential oil by GC- MS

S.No	RT (min)	Name of the compound	Peak Area %
1	3.29	ç-Terpinene	19.28
2	3.86	5-Isopropyl-2-methylbicyclo[3 .1.0]hexan-2-ol	0.68
3	5.16	Terpinen-4-ol	0.43
4	7.08	Phenol,5-methyl-2-(1-methylethyl)-	79.51
5	7.92	2-Cyclohexen-1-one, 3-methyl-6 (1-methyl ethylidene)-	0.11

The phenol identified in *Trachyspermum ammi* seed essential oil with peak area percent of 79.51 was widely used as a starting material in the preparation of plastics, explosives, drugs. At low concentration, used as a disinfectant in mouthwash and household cleaners. According to British surgeon Joseph Lister, phenol reduces the mortality rate to 15% from 45% when applied in their laboratory for disinfection. In addition, it also possess anti-insecticidal (Chaubey, et.al.2008) antimicrobial, (Gilani, et.al., 2013) analgesic, anti-nociceptive (Chahal, et.al., 2017) properties. Terpinenehaving anti-inflammatory activitywas obtained with peak area percent of 19.28.(Theresa Raquel , et.al., 2015)

3.3 Antibacterial activity of essential oil from *Cinnamomum tamala* bark and *Trachyspermum ammi* seed.

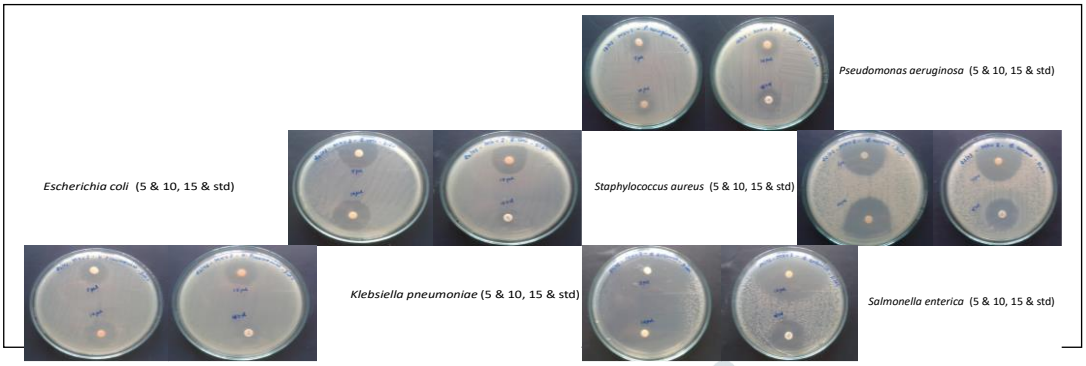


Figure. 3 Showing Antibacterial activity of essential oil from *Cinnamomum tamala* bark

The antibacterial activity was tested against *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella enteric*.The zone of inhibition at studied concentration 5, 10,15µl was high with *S. enteric*,*S. aureus*,*E.coli, K.pneumonia, P. aeruginosa* when compared with standard chloramphenicol. The antimicrobial activity of essential oil was mainly due to the disruption of bacterial cell wall leading to the membrane lysis, leakage of intracellular contents causing cell lysis. (Zhang, et.al., 2016) The presence of alkaloids, terpensin the essential oil of *Cinnamomumtamala*barkaids antimicrobial activity by stopping pathogenic bacteria's normal growth. (Skandamis, et.al., 2001), (Carson, et.al., 2002).

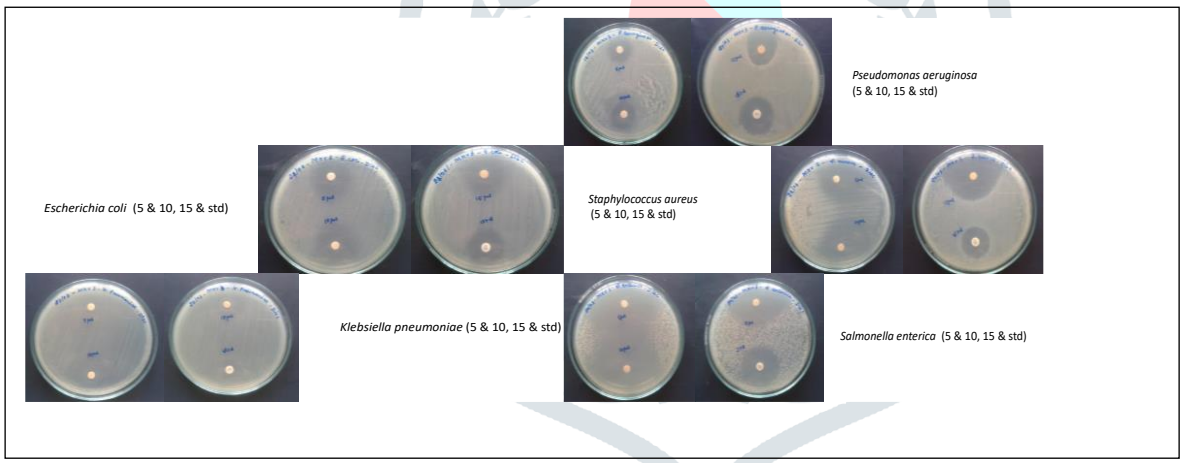


Figure.4 Showing Antibacterial activity of essential oil from *Trachyspermum ammi* seed

The antibacterial activity was high with*Salmonella enteric, Staphylococcus aureus, Escherichia coli,Pseudomonas aeruginosa*at studied concentration 5, 10,15µland the zone of inhibition obtained was low with *Klebsiella pneumonia* when compared with standard. The components present in essential oil induces antibacterial activity by damaging the integrity of membrane through altering pH, inorganic ion equilibrium. (Delgado, et.al.,2004) The antimicrobial activity was good with both gram positive and gram negative bacteria which might be due to the phenolic compounds in essential oil that could melt microbial membrane and generate openings for antibacterial function.(Ultee, et.al., 2002)

3.4 Biochemical studies

Table. 3 Showing results of biochemical parameters studied in essential oil Extracted from *Cinnamomum tamala* bark and *Trachyspermum ammi* seed

Parameters studied	*Cinnamomum tamala* bark (mg/g)	*Trachyspermum ammi* seed(mg/g)
Secondary metabolites		
Total phenolics	0.21±0.01	0.29±0.02****
Total flavonoid	0.15±0.03	0.19±0.03*
Antioxidant activity		
Phosphomolybdenum	0.19±0.08	0.33±0.03**
Nitric oxide scavenging	0.48±0.02****	0.27±0.05
Reducing power	0.20±0.04	0.37±0.03****
Metal chelating	0.11±0.03	0.15±0.02*
Phytonutrient assay		
Carbohydrate	0.11 ± 0.04^{NS}	0.11 ± 0.01^{NS}
Protein	0.27±0.01****	0.23±0.01
Aminoacids	0.04 ± 0.01^{NS}	0.04 ± 0.01^{NS}

$P \leq 0.05$-*, $P \leq 0.01$-**, $P \leq 0.001$ -***, $P \leq 0.0001$- ****, NS-Non Significant

The secondary metabolites, antioxidant activity, phytonutrients were studied for essential oil extracted from *Cinnamomum tamala*bark and *Trachyspermum ammi* seed. The phenolic and flavonoid content was high with *Trachyspermumammi*seedessential oil when compared to essential oil from *Cinnamomum tamala* bark.The phosphomolybdenum, reducing power, metal chelatingactivity was high with essential oil from *Trachyspermum ammi* seed.While,nitric oxide scavenging activity was high with essential oil extracted from *Cinnamomum tamala* bark. The protein content was high with essential oil from *Cinnamomum tamala* bark.Whereas, the carbohydrate and aminoacid content was found to be similar in both the samples studied. Presence of terpene, phenol, other volatile compounds allows essential oil to behave as a naturalantioxidants.(Fellah, et.al., 2006), (Ahmed, et.al., 2007) which help in neutralizing free radical and reduces oxidation induced stress causing damage to DNA, protein, membrane thereby preventing cancer and vascular disease.(Halliwell, 1997), (Nakayama, et.al., 1993) Likewise, phenolic compounds redox properties allows absorption, neutralization,quenching of peroxides, thus imparting antioxidant activities.(Jamuna, et.al., 2017)

3.5 Methyl Thiazol Tetrazolium (MTT) assay

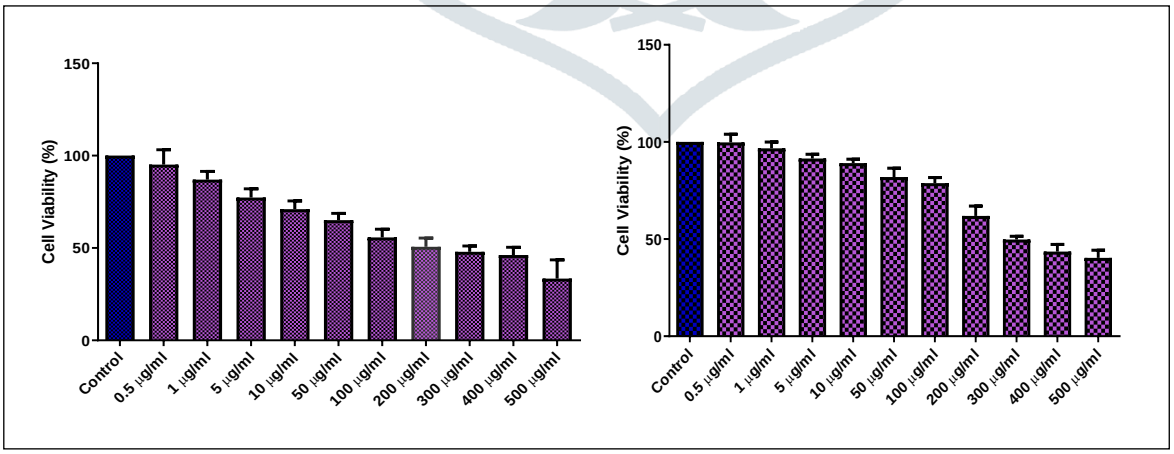
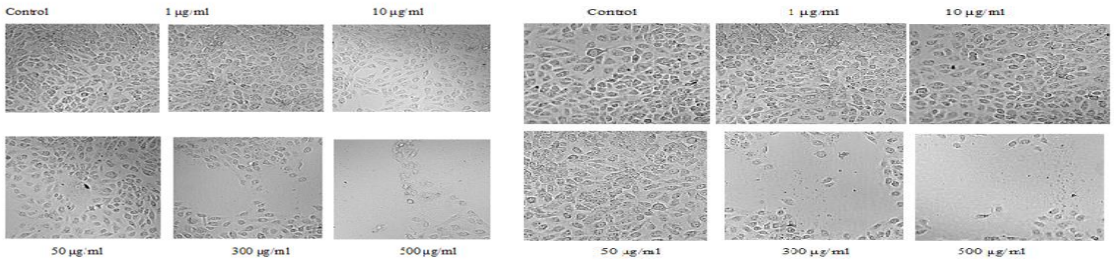


Figure.5A. Showing Cell viability (%) against human oral squamous carcinoma cell line HSC 3 studied with essential oil extracted from *Cinnamomum tamala* bark. B. Showing Cell viability (%) against human oral squamous carcinoma cell line HSC 3 studied with essential oil extracted from *Trachyspermum ammi* seed.

MTT assay (cytotoxic test) was performedto study the dehydrogenase activity in mitochondria to assess the cell viabilityin human oral squamous carcinoma cells.The viability of cells tested with essential oil extracted from *Cinnamomum tamala* bark at different concentrations 0.5, 1, 5, 10, 50,100,200,300,400,500µg/mlwas found to

be 95.16, 86.99,77.29, 71.06, 65.09, 55.76, 50.63, 47.91, 46.19, 33.45%. The IC 50 value was found to be 32.24µg/mlfor essential oil from *Cinnamomum tamala* bark.While, for *Trachyspermum ammi* seed it was 99.74, 96.69, 91.45, 89.09, 81.93, 78.79, 61.95, 49.76, 43.60, 40.34%. *Trachyspermum ammi* seed essential oil showed IC50 value of 112.5(Fig.5A and B). The variation in IC50 value predicts drug efficacy and the observed variation might be due to essential oil that could bring changes in the mitochondrial enzyme activity leading to cell death.



A B

Fig. 6A Showing few images of human oral squamous carcinoma cell line HSC-3 and cells at different concentration of essential oil from *Cinnamomum tamala* bark. B. Images of control HSC 3 cells and cells at different concentration of essential oil from *Trachyspermum ammi* seed. (MTT Assay)

The HSC 3 cells on treatment with essential oil extracted from *Cinnamomum tamala* bark and *Trachyspermum ammi* seed demonstrates reduced cell number, cell shrinkage at higher concentrations. (Fig 6A &B).The obtained results from the present study, confirm it to be a good anticancer, antibacterial agent.

4.Conclusion

The author would like to conclude that based on the obtained results the anticancer activity was found to be good with *Cinnamomum tamala* bark as its IC 50 value was 32.24µg/ml. Both showed very good antibacterial activity.

5.Acknowledgment

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