ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com
Journal DOI: 10.21474/IJAR01

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Phytochemical analysis and Biological studies of Indian medicinal plants *Myristica fragrans* and *Tinospora cordifolia.*

Jhorar Ramesh[1], Kaur Amanpreet[1], *Mukherjee kumar Tapan[1] and Batra Priya[2].

1. Department of Biotechnology, Research Scholar of biotechnology, Maharishi Markandeshwar University, Mullana (Ambala).
2. Department of Microbiology, Faculty of Microbiology, Punjab Agriculture University, Ludhiana.

Manuscript Info

Manuscript History:

Received: 17 March 2016
Final Accepted: 26 April 2016
Published Online: May 2016

Key words:
Phytochemicals, Antimicrobial activity, Antioxidant activity, Medicinal plants.

Corresponding Author

Mukherjee kumar Tapan.

Abstract

Phytochemical screening, isolation, antimicrobial and antioxidant activities of methanol, ethanol chloroform and water crude extracts of *Myristica fragrans* and *Tinospora cordifolia* were studied in this work. The aim of this work is to ascertain the level of antimicrobial activity and antioxidant properties of different solvent extracts of *M. fragrans* and *T. cordifolia* as well as isolated phytochemicals. The preliminary screening of various extracts was carried out using *E. coli, C. sakazakii* and *P. aeruginosa* by agar well diffusion method. The DPPH method used to determine the antioxidant potential of plant extracts and isolated phytochemicals. Myristicin and eugenol present in methanolic extracts exhibit direct relation with antimicrobial and antioxidant activity while palmatine and berberine could be responsible for *T. cordifolia* activities. The result showed that *M. fragrans* and *T. cordifolia* extracts have pharmocological active compounds with great antioxidant and antimicrobial effects. The results obtained from this study justify the use of this plant in traditional folk medicine and provide leads which could be further exploited for development of new and potent antimicrobials.

Introduction:-

The search for natural origin products with pharmacological properties has significantly contributed to the discovery of new substances that have important uses in medicine. In this sense India stands out as a potential reservoir of phytochemicals for having vast plant diversity and also most of plants are unexplored relative to their pharmacological potential (Kala et al., 2006). In the middle of the many families of plants investigated, the *Myristicaceae* and Menispermaceae deserve some special interest. *Tinospora cordifolia* (*T. cordifolia*) commonly known as 'giloy' belongs to *Menispermaceae* family and *Myristica fragrans* (*M. fragrans*) commonly known as nutmeg belongs to family *Myristicaceae,* well known medicinal plants in Indian traditional folk medicine. Taking account of the vast prospective of plants of their antimicrobial as well antioxidative properties, systematic investigation was commenced to screen the local Indian flora for their antimicrobial as well antioxidative properties from *M. fragrans* and *T. cordifolia.* Extracts from kernel of *M. fragrans* and stem of *T. cordifolia* are extensively used in folk medicine for infectious, inflammatory and oxidative stress conditions (Hinneburg et al., 2006; Yano et al., 2006; Tajjudin et al., 2003; Jayaprakash e al., 2015).

Analysis of the crude extract of *M. fragrans* using gas chromatography coupled to mass spectrometry (GC/ESI-MS) indicated the presence of alkyl benzene derivatives (myristicin, elemicin, safrole, etc.), trimyristin, mace-lignan, terpenes, myristic acid, neolignan, β-pinene and α-pinene (Pandey et al., 2015; Qiu et al., 2004). 2,7-Diphenyl-1,6-dioxopyridazino(4,5:2',3')pyrrolo, Propanoic acid, (4',5'-d)pyridazine, hydroxymethylcolchicines, palmatine and berberine were reported from the stem extracts of *T. cordifolia* (Albinjose et al., 2015). *T. cordifolia* well known as giloy has shown antispasmodic, antipyretic, antineoplastic, hypolipidemic, hypoglycemic, immunopotentiating, and

hepatoprotective properties (Sing et al., 2003; Jagetia et al., 2006; Adhvaryu et al., 2007). The plant extracts and metabolites of *M. fragrans* have been shown to have hypolipidemic, hypocholesterolemic, hepatoprotective, anti-depressant, anti-bacterial, aphrodisiac, memory enhancer, property, anticarcinogenic, and anti-inflammatory activities (Sonavane et al., 2002; Zaidi et al., 2009; Hussain et al., 1991; Ozaki et al., 1989).

Previous studies have revealed the individual reports regarding the antibacterial, antifungal and antioxidant activities of *M. fragrans* and *T. cordifolia* but lack specific activity of particular plant part and its constituents regarding phytochemical analysis, antimicrobial and antioxidant activity. In our continuing efforts for finding new effective antioxidant and anti-microbial agents from natural sources, we have investigated the stem extracts of *T. cordifolia* and kernel of *M. fragrans* for their bioactive constituents. For the first time an attempt was made to evaluate the antimicrobial potential of constituent of *M. fragrans* and *T. cordifolia* plants against food borne pathogen *C. sakazakii* which is main causative agent of meningitis and had 40% mortality rate (Lai, 2001; Caubilla-Barron et al., 2007). *E. coli*, being more significant among other food borne pathogens due to its severe consequences of infection affecting all age groups, distinctive acid tolerance and low infectious dose causing, is being included in the study. *P. aeruginosa* is another food borne pathogen mostly affecting people with compromised host defense mechanisms and mostly colonizing the lungs of patients with cystic fibrosis in both young as well as adults (Lim et al., 2010; Yoon et al., 2002).

In our study we try to find out the most active constituent of plant extract against selected food borne pathogens. In this study, therefore, various experiments performed for investigating a range of concentrations of crude extract from *M. fragrans* and *T. cordifolia* plants and the individual purified fractions were conducted to evaluate the potential for development of new natural antibacterial medicine. The antimicrobial properties of these plant extracts that target cellular viability of bacteria has been adequately discussed previously, but very little research have highlighted the effects of their purified compounds in modulating various aspects of bacterial virulence, critical for pathogenesis in the host. The overall objective of study was to ascertain the bioactivity of extracts and fraction of plants on selected food borne pathogens and identify the probable compound present in fraction.

Materials and Methods:-

Bacterial Strains and Chemicals:-

Standard strains of the following organisms were used in this study procured from Institute of Microbial technology (IMTECH) Chandigarh in lyophilized powder form. They included: *Escherichia coli (*MTCC-443), *Cronobacter sakazakii (*MTCC-2958) and *Pseudomonas aeruginosa (*MTCC-424). These organisms are food borne pathogens and leading cause of acquired infections and high mortality rate. They were selected on their prevalence and increase in the resistance against standard antibiotics. DMSO and Silica gel 60 were obtained from Merck, TTC. Distilled water, hexane, benzene, acetonitrile, ethyl acetate, ethanol, methanol, chloroform, formic acid and ascorbic acid were obtained from HiMedia. Mueller Hinton Agar media and Mueller Hinton Broth were also obtained from HiMedia. DPPH was obtained from Sigma-Aldrich.

Resuscitation of Bacterial strain:-

Bacterial isolates of test organisms were obtained from stock cultures maintained at our laboratory. Resuscitation of the cultures was done by inoculating the organisms on Mueller Hinton Agar (MHA) and plates incubated at 37 °C for 24 hours. They were frequently sub-cultured on MHA or Nutrient agar slants and stored in the refrigerator.

Preparation of plant extract:-

The plant materials were dried for one week and then powdered with the help of mortar and pestle and preserved in air tight bottles for further use. 25 gm of plant sample was used for extraction and placed in the thimble of Soxhlet. The solvents were Methanol, Ethanol, Chloroform and Water used for extraction. The soxhlet was running for 48 hrs and solvent mixed extract collected in round bottom flask. Filtrate was evaporated to near dryness to obtain a final concentration. For further studies, the extract was reconstituted with 10 ml of solvent. Each solution was stored at 4°C in sterilized tubes until further use.

Antimicrobial Susceptibility Testing:-

Sensitivity testing of *M. fragrans and T. cordifolia* extracts was done using the agar well diffusion method previously described by Irobi et al 1994(Irobi et al., 1994). The bacterial isolates were grown in Muller Himlton Agar (MHA). Plates were inoculated with the organisms prepared at 0.5 McFarland standards. Wells were bored into the agar medium using sterile 6 mm cork borer. Four holes were bored in one plate. The first two wells were

filled with solution of the extract at concentrations of 100 and 50 mg/mL. The other two wells were filled with a positive control (ciprofloxacin 1.25 mg/mL) and negative control [dimethylsulfoxide (DMSO)]. For proper diffusion of the solution into medium the plates were kept in Laminar Air follow for 20 minutes before incubation. Plates were incubated for 24 hrs at 37^0C. After 24 hrs zone of inhibition was observed and evaluated by measuring diameter of Zone of inhibition. The experiment was replicated three times and zones of inhibition reported as mean ± SD. That extract which gives maximum zone of inhibition was chosen for Minimum Inhibitory Concentration.

Determination of the Minimum Inhibitory Concentration of the Plant Extracts:-

The Minimum Inhibitory Concentration (MIC) of Methanolic extracts of *M. fragrans* and *T. cordifolia* against food borne pathogens were determined by using two fold broth microdilution method of 96 wells microtiter plate described by Njume et al 2011 (Njume et al., 2011). Two stock solutions of methanolic extracts at a concentration 40 mg/ml were prepared with DMSO. All the extracts mixed properly and sterilized by using 0.45 µm membrane filter paper. Approximately 50µl of each extract was added to the first well containing 50 µl of Muller Hinton broth (MHB) and serial diluted by two fold method, resulting in final concentration raging from (40 mg/ml to 0.0781mg/ml). Subsequently 50 µl of bacterial suspension containing $1\text{-}2x10^8$ CFU/ml was added into the 1st and 11th well. The 11^{th} well served as control well and 12^{th} well as negative control. The plates were incubated for overnight at 37^0 C. The procedure was repeated thrice for each extract. For detection of MIC value dye indicator is used. For Gram negative 40 µl of 2,3,5 triphenyltetrazolium chloride (TTC) used. After addition of this dye plate was stored for 2 hrs in dark condition. In presence of bacteria TTC is reduced to red formazan which is directly proportional to the viable active cells.

Determination of the Antioxidative Properties of the Plant Extracts:-

The scavenging activity of *M. fragrans* extracts and *T. cordifolia* bark extracts was determined using DPPH **(**2,2-diphenylpicrylhydrazyl**)** assay with some minor modifications described by bolis (Albu et al., 2004). This method depends on the reduction of purple DPPH (Sigma-Aldrich) to a yellow colored diphenylpicrylhydrazine. The determination of the disappearance of free radicals was done using a spectrophotometer. The remaining DPPH which showed maximum absorption at 517nm was measured. Each plant extract sample's stock solution (10 mg/ml) was diluted to final concentrations of (9, 8, 7, 6, 5, 4, 3, 2 and 1 mg/ml) in DMSO (Merck). 1 ml of a 0.3 mM DPPH was added to 2.5 ml of sample solution of different concentrations. The antioxidant ascorbic acid (HiMedia) was used as positive control and prepared in the same manner as above. As DPPH is sensitive to light, it is exposed to the minimum possible light. These solutions were allowed to react at room temperature (RT) for 30 minutes. The absorbance values were measured at 518 nm and converted into the percentage antioxidant activity using the following equation:

$$\text{Free radical scavenging activity (\%):} \quad \frac{\text{Abs (control)} - \text{Abs (sample)} \times 100}{\text{Abs (control)}}$$

Where, (Abs = Absorbance)

IC_{50} value was calculated using plotting graph in excel that represent at which minimum 50% inhibition is done.

Phytochemical fractionation and antimicrobial analysis:-

Column Chromatography:-

Column chromatography was used as a purification technique. A glass column was prepared by packing the column with slurry of silica gel having 60-120 mesh size. A 40 cm long and having 2.5 cm diameter was used for separation of compounds. The mixture was then loaded onto the above mentioned silica gel column and simultaneously equilibrated with different elution solvents and mixture of solvents mainly hexane, benzene, ethyl acetate, formic acid, toluene and methanol. The eluted sample collected and concentrated on rotary evaporator to remove the excess solvent.

Determination of Minimum Inhibitory Concentration of fractions:-

The Minimum Inhibitory Concentration (MIC) of the isolated fractions was evaluated by two fold micro-broth dilution method performed in 96-well plate as previously described for the extracts; active fractions were further analyzed to determine the purity. Ciprofloxacin was used as a positive control at 1.25 mg/ml. An ELISA microplate reader adjusted to 620 nm was used to measure the absorbance of the plates before and after incubation at 37 °C. The absorbencies were compared to detect an increase or decrease in bacterial growth. The lowest concentration of the fraction resulting in inhibition of bacterial growth was recorded as the MIC by ELISA microreader plate.

Gas chromatography-Mass Spectrometry analysis of Fractions:-

Gas chromatography-Mass Spectrometry (GC-MS) technique was used in this study to identify the components present in the extracts of the test plant. GC-MS technique was carried out at NIPER, Mohali. Experimental condition of GC-MS system was as silica capillary column was used. Mobile phase was helium gas and flow rate 1ml/min operating at electron impact mode. The injection volume of sample was 0.5µl, injector temperature 250^0 and ion source temperature 280^0C. The oven temperature was programmed at 40^0C raised upto 250^0C. Mass spectra were taken at 70 eV at a 0.5 seconds intervals and running time set 50 minutes for samples.

Results:-

Susceptibility Testing:-

The zones of inhibition (clear zones on agar) for all three microorganisms were measured in mm at different concentration and the breakpoint for susceptibility was taken as ≥11 mm. **Table 1** showed the antimicrobial activity of various extracts of *M. fragrans* against three selected gram negative foodborne pathogens. The methanolic extract of *M. fragrans* showed satisfactory antimicrobial activity against all three pathogens. Maximum antimicrobial activity of methanolic extract of *M. fragrans* was observed against E.coli (25±0.7) and lowest detected against p. aeruginosa (18±1.3). The ethanolic exract of *M. fragrans* was less potent than the methanolic extract and showed highest activity against E. coli (24±1.1) followed by C. sakazakii (19±1.9) and P. aeruginosa (18±1.3). While antimicrobial activities of chloroform extracts of *M. fragrans* was slightly lowered as compared to the other extracts. Chloroform shows highest antimicrobial activity against E. coli (22±2.3) followed by P. aeruginosa (19±2.3) and C. sakazakii (15±1.3). While water extract demonstrated poor activity against any of the test organism. The present study strongly supports that *M. fragrans* have strong antibacterial property against all pathogens. The antimicrobial property could be attributed to occurrence of various compounds in extracts (Gupta et al., 2013). In an another study methanolic extract of *M. fragrans* shows inhibitory effect against gram positive and gram negative bacteria that also supports our findings (Sazia et al., 2015).

T. cordifolia extracts exhibited variable inhibitory response against pathogenic bacteria. **Table 2** showed that methanolic extract of *T. cordifolia* highly active against all three tested organism followed by chloroform and ethanolic. E. coli exhibited appreciable sensitivity to methanolic, ethanolic and chloroform extracts with zone of inhibition values of 26mm, 25mm and 23mm respectively at 100 mg/mL. Similarly P. aeroginosa also shows variabile susceptibility against methanolic, ethanolic and chloroform extract. P. aeroginosa was highly sensitive against methanolic extract (25±2.33) and reasonably observant against chloroform extract (25±2.64). Ethanolic extract was least active against P. aeroginosa, while distilled water extract shows no effect. Against C. sakazakii methanol extract of *T. cordifolia* possess highest antimicrobial activity (23±1.52) followed by ethanolic and chloroform extract. Distilled water extract was totally inactive against C. sakazakii and didn't show zone of inhibition. The plant extract was less potent than standard antibiotic ciprofloxacin to which pathogens were highly sensitive. Previous study shows that *T. cordifolia* exhibit strong antimicrobial activity against gram positive and gram negative pathogens. In study conducted by Monali et al 2015, methanolic extract of *T. cordifolia* exhibit strong antibacterial activity against both gram positive and gram negative pathogens (Mishra et al., 2015). Study conducted by Shivam singh et al 2012 revealed that methanolic extract of *T. cordifolia* produces considerable antimicrobial activity (singh et al., 2012).On the basis of finding methanolic extract was selected for fractionation for indentifying the component involved in antimicrobial activity.

Minimum Inhibitory Concentration Determination:-

Minimum inhibitory concentration (MIC) is defined as the highest dilution or least concentration of the extract that inhibit the growth of microorganisms. An antimicrobial agent and it monitors the activity of new antimicrobial agent. MIC of methanolic extract of *M. fragrans* and *T. cordifolia* against food borne pathogens were specified in **Tables 3** and **4**. The low MIC values indicated that the extract has strong antibacterial activity. The result revealed that methanol extracts of *M. fragrans* possess good antibacterial activities against selected food borne pathogens (Table 3).The methanolic extract of *M. fragrans* shows lowest MIC value against *E. coli* (0.15625 mg/ml) followed by *C. sakazakii* and *P. aeruginosa* (0.3125 mg/ml). Study conducted by Lyekhoetin *et al* showed that MIC was higher for *E. coli* of extract of *M. fragrans* (Omoruyi et al., 2012). Our study along with previous studies shows that methanolic extract of *M. fragrans* is mostly active against *E. coli* and *P. aeruginosa*. Antimicrobial activity of methanolic extract against *C. sakazakii* was not reported. The methanolic extract of *M. fragrans* was less potent than the standard antibiotic ciprofloxacin but still shows significant bactericidal activity.

In table 4 antimicrobial activity of *T. cordifolia* has been shown, with methanolic extract of *T. cordifolia* mostly against *E. coli* and *P. aeruginosa* (0.15625 mg/mL). The MIC value for *C. sakazakii* is reported to be 0.3125mg/ml.

Column Chromatography Analysis and Minimum Inhibitory Concentration Determination of Fractions:-
A 40cm long x 2.5 cm diameter glass column was packed to height of 31 cm with slurry of silica gel 60-120 mesh size. The *M. fragrans* extract filled column was eluted with 100% hexane followed by an increasing gradient of benzene starting with 10% upto100% benzene. This was followed with a solvent system of increasing concentration of toluene and ethyl acetate. Total nine fractions (F1-F9) of *M. fragrans* were collected from silica column. Test tube fractions were collected, concentrated and then monitored with TLC and spots were detected by iodine stream.

Similarly, fractionation of methanol soluble partition of *T. cordifolia* by column chromatography on silica gel with gradient using chloroform/methanol and Chloroform, Ethyl acetate and formic acid of increasing polarity afforded eight fractions and subfractions. Fraction 1 (TF1) was chromatographed on silica gel with hexane/acetone gradient to afford one spot. Fraction 3 (TF3) was purified on a column with methanol/water to yield pure compound. Fraction 3 (TF 3) was chromatographed using silica column with a methanol/water and ethyl acetate, formic acid and water gradient to furnish subfractions [TF5-TF6, EFH7 (TF7)-EFH9 (TF9)]. Isolated fraction shows significant antibacterial activity against selected pathogens. Fraction F4 purified from methanolic extract of *M. fragrans* was mostly active against *P. aeruginosa* followed by *E. coli* and *C. sakazakii.* MIC value of all fractionated compounds was shown in the **Table 5** and **6**. Some fractions did not show any activity which means that compound was not participating in antimicrobial property of plant extract. All fractions are subjected to be identified by GC-MS analysis.

Determination of antioxidant activity of the fractions:-
The biological activity of much plant raw material depends on their chemical composition. The DPPH antioxidant activity of the extracts indicates their ability to dispose of hydrogen atoms. The free radical scavenging potency of crude extract as well as isolated fraction is presented in **Table 7, 8** and **9**. As illustrated the TC5 fraction was found to be exhibit the highest radical scavenging activity (IC50-0.016). The DPPH radical scavenging activity of the tested samples were in order of TF4>F4>methanolic extract of *T. cordifolia*>chloroform of *T. cordifolia*.The statistic results have revealed a significant difference of the DPPH antioxidant activity between solvent extracts of the above mentioned plants and their isolated fractions. The results show that the isolated fractions are more active than the crude extract. The increasing interest in the search for natural alternatives for synthetic antioxidants has led to the antioxidant evaluation of a number of plant sources.

Gas chromatography-Mass Spectrometry analysis: -
Gas chromatography (GC) chromatograms of methanolic extract of both plants from the GC system are shown in figure 1 and 2. These extracts were selected based on their purity and their antimicrobial activity against selected food borne pathogens. Although 17 fractions isolated via column chromatography; only four fractions were analyzed by GC-MS. Selection of fractions based on their biological activity. The chromatogram of methanolic extract of *M. fragrans* shows 10 different peaks. Ten major constituents from 10 peaks were identified as difluorodiazene, methyleugenol, benzene, 1,2 dimethoxy-4-(1-propanyl), myristicin, elemicin, methoxyeugenol, isoelimicin, methyl tetradecanoate, tetradecanoic acid and corynan-17-ol,18,19-didehydro-10-methoxy. Similarly GCMS analysis of methanolic extract of *T. cordifolia* shows 8 major peaks. The compounds are difluorozene, vanillin lactoside (glycoside), 5,6-dihydro-9,10-dimethoxybezo[g]-1,3-benzodioxolo[5,6-a]quinolizinium, cordifolioside A, nootkaton-11,12-epoxide, metholene, hexadecanoic acid (palmitic acid), methyl10-trans,12-cis-octadecadienoate. Four fractions F4, F7, TF2 and TF4 show highest antimicrobial and antioxidant activity. Analysis of methanolic fraction of F4 showed high level of 6-allyl-4-methoxy-1,3-benzodioxole commonly known as Myristicin shown in figure 3. F7 fraction presented several peaks; it however had the compound 4-Allyl-2-methoxyphenol (eugenol) identified as the major peak from this fraction shown in figure 4. TF2 and TF4 fractions showed as major compounds hexadecanoic acid (Palmitic acid) and 5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium known as berberine were identified on the GC-MS chromatogram shown in Figure 5 and 6. Most compounds revealed by GC-MS in this study were fatty acid and alkaloids and have been reported to have antibacterial and antioxidant activity. The observed potent antimicrobial and antioxidant property of methanolic extract of *M. fragrans* observed in our study could be likened to the presence of myristcin and eugenol while for biological activity of *T. cordifolia* could be related to palmitic acid and berberine.

Discussion:-

This study has established that the extracts and isolated compounds have moderate antimicrobial activity and significant antioxidant activity and could be effective in treating bacterial mediated diseases and helpful in managing oxidative stress. More specifically, in this study we have investigated the antioxidative and antimicrobial effect of the purified constituents of *M. fragrans* and *T. cordifolia* plant extracts against three foodborne microorganism i.e. *E. coli*, *P. aeruginosa* and *C. sakazakii*.The findings presented here justified the use of *M. fragrans* and *T. cordifolia* plants in folk medicine in India. Based on the qualitative antimicrobial and antioxidant screening using DPPH assay, methanolic extracts of *M. fragrans* and *T. cordifolia* contained antibacterial and antioxidant compounds. The extracts of seed of *M. fragrans* and stem of *T. cordifolia* were chromatographed to provide many combined fractions containing a mixture of aromatic compounds as observed by GC-MS analysis (fig 1 and 2).

Natural products with medicinal value are gradually gaining importance in clinical research due to their well-known property of no side effects as compared to drugs. *Tinospora cordifolia and Myristica fragrans* are known for their immense application in the treatment of various diseases in the traditional ayurvedic literature. Recently researcher identifies many phytochemical of these plants and their role in disease control has led to active interest to use these plants as medicinal across the globe (Shafiei et al., 2012; Hou et al., 2012; Sharma et al., 2012; Patel et al., 2011; More et al., 2012). This study mainly focus on isolation bioactive component of plant extract due which a crude extract show biological activity and their role in disease control.The future scope of this study remains in exploiting the biochemical and signaling pathways affected by the compounds isolated from *Tinospora cordifolia* and *Myristica fragrans* so as to enable new and effective formulation in disease eradication. In this study ciprofloxacin used as a standard antibiotic because initially ciprofloxacin was used to treat infection caused by gram negative bacteria and here we used gram negative bacteria. Increasing ciprofloxacin consumption particularly in ICU leads to selection of resistant mutants among nosocomial pathogens since fluoroquinolones are particularly greater selectors of resistant among aminoglycoside, carapenems or b-lactams and these strains resistant can more easily spread than strain resistant to other drugs (Fasugba et al., 2015).

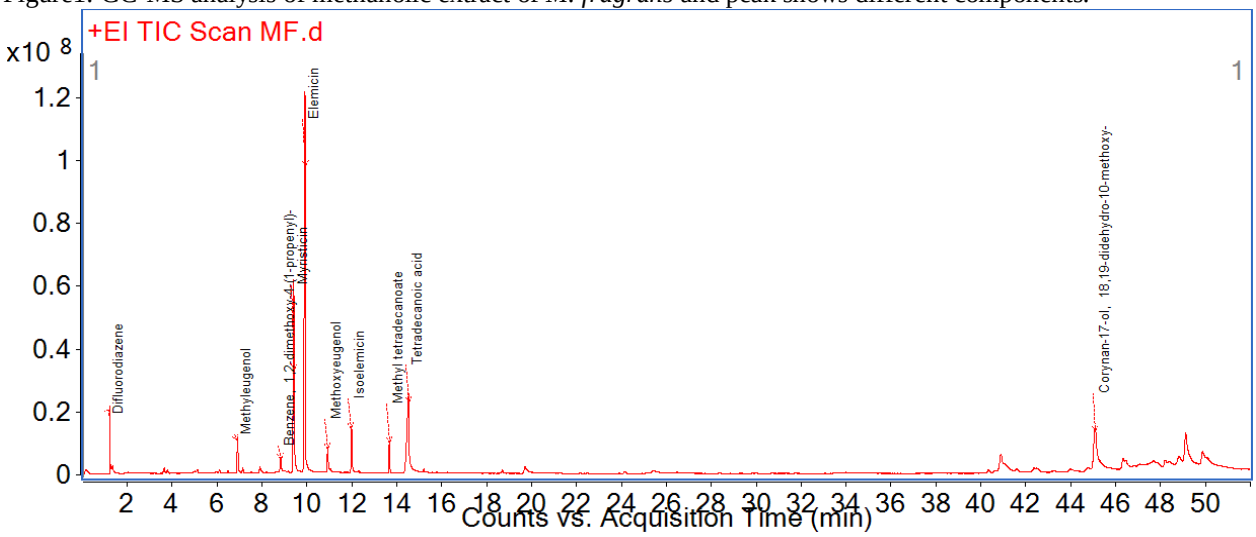
Extensive repeated isolation and purification by column chromatography afforded bioactive compound namely myristicin, eugenol and elimicin from methanolic extract of *M. fragrans.* Previously these components isolated from other medicinal plants such as *Syzygium aromaticum*(clove), *Heracleum transcaucasium* and *Heracleum anisactis* shows significant antimicrobial activity (Devi et al., 2010; Torbati et al., 2014).

The antimicrobial activities of phenolic compounds may involve multiple modes of action. In studies it proven that plant essential oil degrade the cell wall of bacteria, disrupt cytoplasmic membrane by interacting with composition, damaging membrane proteins, modulate action of membrane integrated enzymes and cause leakage of cellular components, cytoplasm coagulation, depletion of proton motive force, change constituents of phospholipids and fatty acid, halt the energy production and metabolism, alter nutrient uptake and ATP synthesis, regulate DNA synthesis and RNA synthesis and destroy protein translocation and mitochondrial action in eukaryotes (Lambert et al., 2001; Pandey et al., 2012; Raccach, 1984).

Biological activity of *M. fragrans* showed that its methanolic extract displayed antimicrobial activity. However, most fractions isolated from *M. fragrans* showed inhibitory effect against *E. coli, C. sakazakii* and *P. aeruginosa* while fraction F1 and F9 did not show any effects against *E. coli.*In addition F4 and F7 fraction displayed antimicrobial activity against these food borne pathogens. F4 shows lowest MIC value against *P. aeruginosa* while F7 mostly active against *E. coli.* Both these fractions display significant activity against *C. sakazakii.* F4 and F7 fractions were identified as eugenol and myristicin by GC-MS analysis. In previous study crude extract of *M. fragrans* studied against gram positive (Shafiei et al., 2012) while we investigated the activity of the isolated components of extracts against gram negative. Narasimhan and Dhake have reported trimyristin and myristicin as chief antibacterial principles isolated from nutmeg (Narasimhan et al., 2006). Three lignans, erythro-austrobailignan-6, meso-dihydroguaiaretic acid and nectandrin-B have been isolated from the methanolic extract of nutmeg which were reported to have antifungal activity (Cho et al., 2007).Takikawa *et al* also reported antimicrobial activity of nutmeg against entero-hemorrhagic *E. coli* O157 and it was believed that E. coli O157 highly sensitive to β-pinene (Takikawa et al., 2002). It has been exhibited that lignans and glycosides of *M. fragrans* are metabolized into catechol structure which is biological active and responsible for high antioxidant potential of nutmeg (Nakai et al., 2003). Catechol structure can easily donate phenolic hydrogen or electron to acceptor molecules which makes it powerful antioxidant compound (Shan et al., 2005).

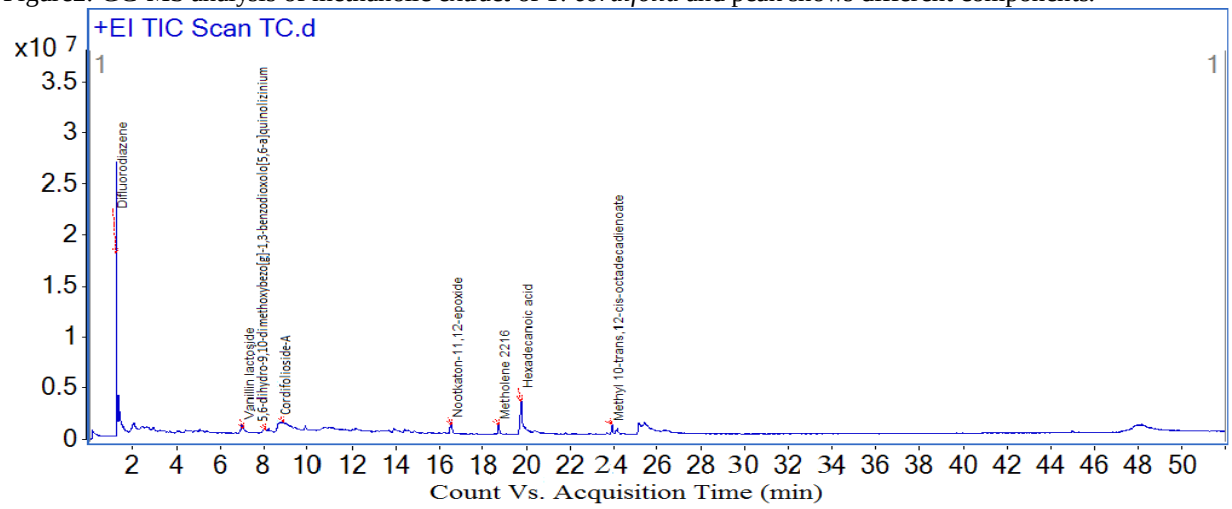
Our study reveals that *T. cordifolia* possesses antimicrobial as well as antioxidant properties supports by previous study conducted by Bonvicini *et al* shows antibacterial activity of ethanolic extract of *T. cordifolia* (Bonvicini et al., 2014). Isolated fraction from methanolic extract of TF2 and TF4 shows moderate MIC value against selected food borne pathogen. These fractions were identified as palmitic acid and berberine by GC-MS analysis. Many compounds have been isolated from *T. cordifolia* which are of antimicrobial and antioxidant properties. The biological activity of palmitic acid and berberine isolated from *T. cordifolia* not yet fully explored. Palmatine, an active component of *T. cordifolia* shows antioxidant properties and therefore plays an important role in preventing oxidative stress (Kim et al., 2009). It has been suggested that anti-microbial as well as antioxidant potential of plant extract could be attributed to major and minor components, it is possible that biological activities of plant crude extract are regulated by interaction between the minor and major components.

Figure1: GC-MS analysis of methanolic extract of *M. fragrans* and peak shows different components.



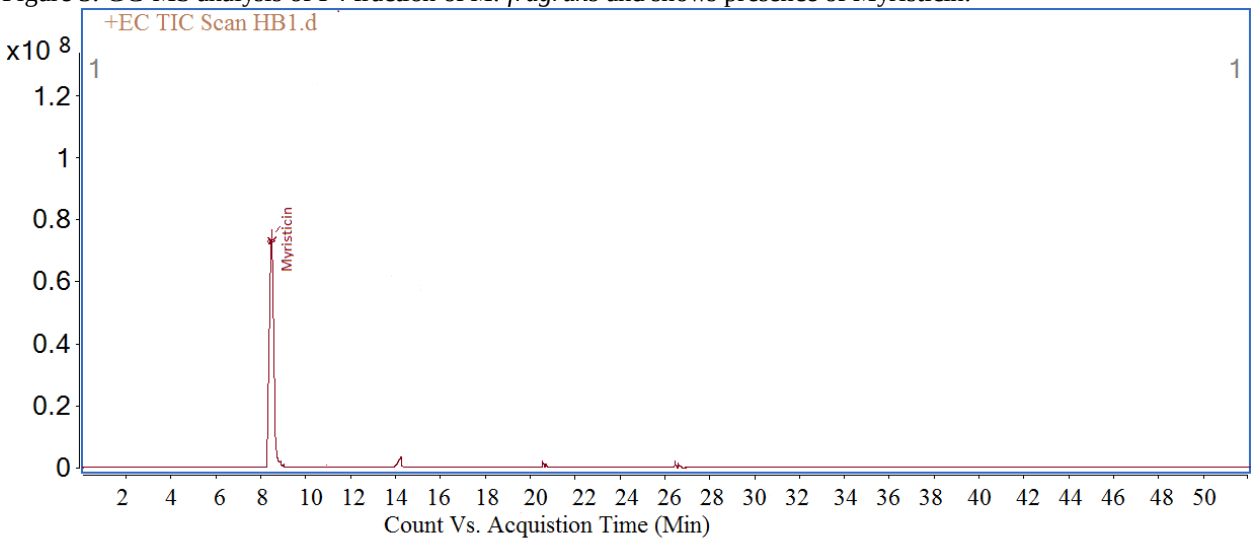
Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Figure2: GC-MS analysis of methanolic extract of *T. cordifolia* and peak shows different components.



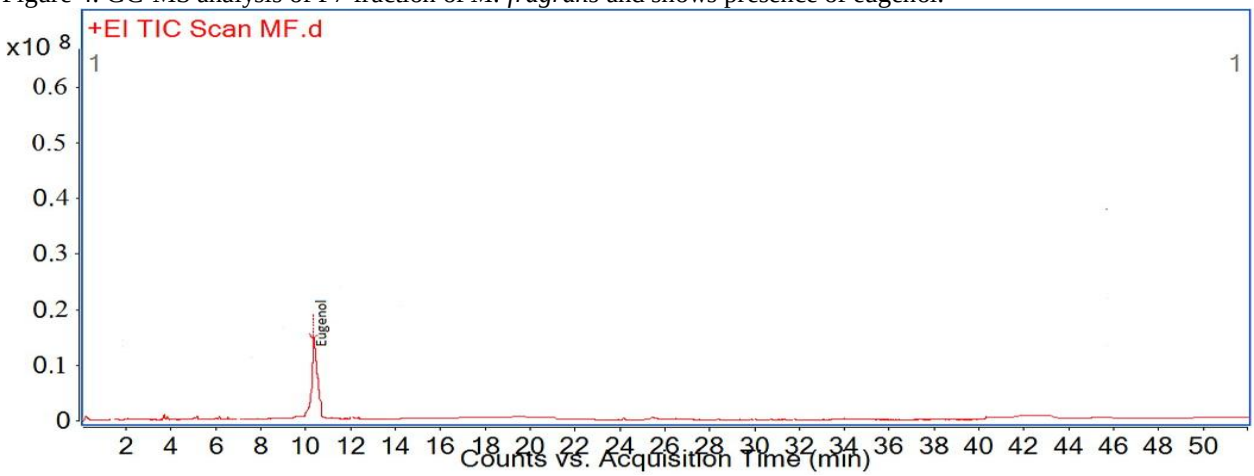
Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Figure 3: GC-MS analysis of F4 fraction of *M. fragrans* and shows presence of Myristicin.



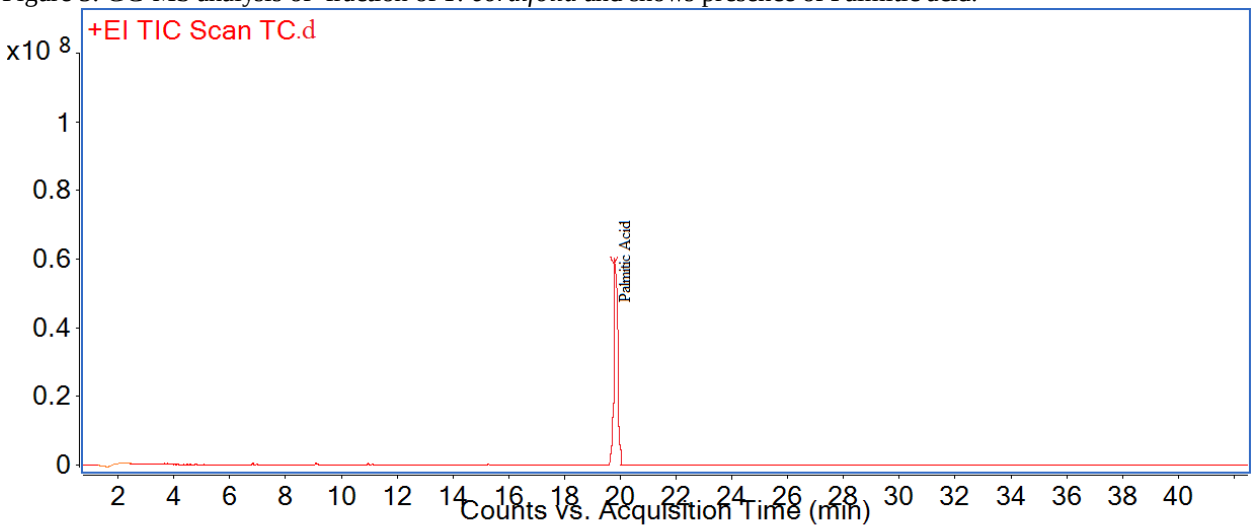
Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Figure 4: GC-MS analysis of F7 fraction of *M. fragrans* and shows presence of eugenol.



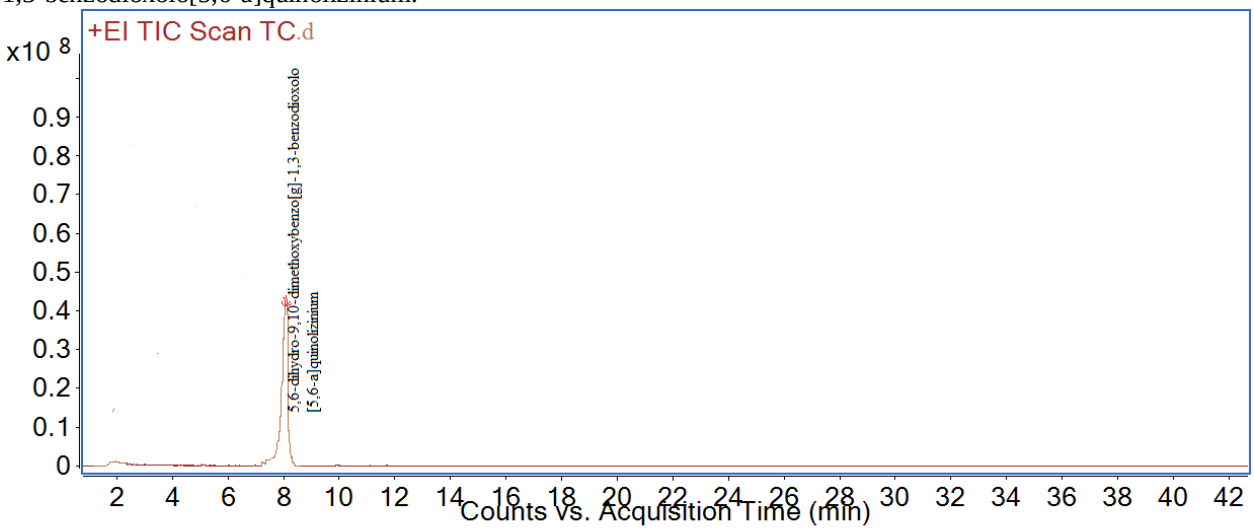
Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Figure 5: GC-MS analysis of fraction of *T. cordifolia* and shows presence of Palmitic acid.



Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Figure 6: GC-MS analysis of fraction of *T. cordifolia* and shows presence of 5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,6-a]quinolizinium.



Gas Chromatography conditions: column: Silica Capillary column, Carrier gas: Helium, Injection volume: 0.5 µl, oven temperature: 250^{0}C, Ioniztion method: Electron ionization (EI), flow rate: 1ml/min and running time: 50 minutes.

Table 1: Zone of inhibition ± SD (mm) of the fruit extracts of *M. fragrans* and ciprofloxacin (1.25) (mg/mL) against organisms.

Organisms	Extracts	Zone of inhibition (mm) at different condition (mg/ml)		
		50 mg/ml	100 mg/ml	Ciprofloxacin
E. coli	Methanol	21 ± 1.3	25 ± 0.7	25
	Ethanol	19 ± 0.6	24 ± 1.1	25
	Chloroform	19 ± 0.8	22 ± 2.3	25
	Distilled Water	0	0	25
C. sakazakii	Methanol	18 ± 1.5	21 ± 1.3	22
	Ethanol	10 ± 2.3	19 ± 1.9	22
	Chloroform	0	15 ± 1.3	22
	Distilled Water	0	0	22
P. aeruginosa	Methanol	18 ± 1.3	21 ± 1.1	25
	Ethanol	18 ± 1.5	18 ± 1.3	25
	Chloroform	14 ± 1.6	19 ± 2.3	25
	Distilled Water	0	0	25

Agar well diffusion method used for antimicrobial activity and calculating zone of inhibition (in mm); standard ciprofloxacin (1.25 mg/ml). Values are mean of triplicate reading (Mean ± Standard Deviation).

Table 2. Zone of inhibition ± SD (mm) of the fruit extracts of *Tinospora cordifolia* and ciprofloxacin (1.25) (mg/ml) against organisms.

Organisms	Extracts	Zone of inhibition (mm) at different condition (mg/mL)		
		50 mg/ml	100 mg/ml	Ciprofloxacin
E. coli	Methanol	19±0.57	26±2.64	25
	Ethanol	18±2.08	25±2.08	25
	Chloroform	17±1.15	23±0.57	25
	Distilled Water	0	0	25
C. sakazakii	Methanol	16±0.57	23±1.52	22
	Ethanol	16±1.0	21±2.08	22
	Chloroform	13±1.52	20±1.15	22
	Distilled Water	0	0	22
P. aeruginosa	Methanol	19±2.6	25±2.33	25
	Ethanol	18±1.52	23±0.57	25
	Chloroform	18±1.33	25±2.64	25
	Distilled Water	0	0	25

Agar well diffusion method used for antimicrobial activity and calculating zone of inhibition (in mm); standard ciprofloxacin (1.25 mg/ml). Values are mean of triplicate reading (Mean ± Standard Deviation).

Table 3: Minimum Inhibitory Concentration of methanol extracts of *Myristica fragrans* and ciprofloxacin on test organisms.

Organisms	Extracts	MIC values (mg/ml)	MIC values of ciprofloxacin (mg/ml)
E. coli	Methanol	0.15625	0.078125
C. sakazakii	Methanol	0.3125	0.15625
P. aeruginosa	Methanol	0.3125	0.078125

Minimum Inhibitory concentration (MIC) calculated by two fold dilution method using 96 wells microtitre plate. MIC values are calculated by observing change in the 2,3,5 triphenyltetrazolium chloride (TTC) dye. Lowest MIC values possess strongest activity. Ciprofloxacin used as a standard antibiotic for comparison.

Table 4: Minimum Inhibitory Concentration of methanol extracts of *Tinospora cordifolia* and ciprofloxacin on test organisms.

Organisms	Extracts	MIC values (mg/ml)	MIC values of ciprofloxacin (mg/ml)
E. coli	Methanol	0.15625	0.078125
C. sakazakii	Methanol	0.3125	0.15625
P. aeruginosa	Methanol	0.15625	0.078125

Minimum Inhibitory concentration (MIC) calculated by two fold dilution method using 96 wells microtitre plate. MIC values are calculated by observing change in the 2,3,5 triphenyltetrazolium chloride (TTC) dye. Lowest MIC values possess strongest activity. Ciprofloxacin used as a standard antibiotic for comparison.

Table 5: Fractionated compounds from *M. fragrans* and MICs in mg/mL against test organisms.

	F1	F2	F3	F4	F5	F6	F7	F8	F9
E. coli	ND	2.5	1.25	0.15625	2.5	0.625	0.078125	0.625	ND
C.sakazakii	ND	ND	2.5	0.3125	0.3125	0.625	0.15625	0.625	ND
P.aeruginosa	2.5	1.25	1.25	0.078125	0.3125	0.3125	0.078125	ND	ND

Minimum Inhibitory Concentration (MIC) of fractionated compounds of *M. fragrans* using two fold dilution methods using 96 wells microtiter plate calculated in mg/ml.

Table 6: Fractionated compounds from *T. cordifolia* and MICs in mg/mL against test organisms.

	TF1	TF2	TF4	TF5	TF6	TF7	TF8	TF9
E. coli	0.15625	0.078125	O.3125	0.078125	0.625	0.625	ND	ND
C. sakazakii	0.625	0.15625	1.25	0.078125	0.3125	0.625	ND	ND
P. aeruginosa	0.625	0.078125	0.15625	0.078125	0.625	1.25	1.25	ND

Minimum Inhibitory Concentration (MIC) of fractionated compounds of *T. cordifolia* using two fold dilution methods using 96 wells microtiter plate calculated in mg/ml.

Table 7: IC_{50} value of various extracts of *M. fragrans* by DPPH method:

Plant Extracts	IC_{50} value of extracts	IC_{50} value of ascorbic acid
Methanol	3.29±0.36	0.07±0.01
Ethanol	4.58±0.18	0.07±0.01
Chloroform	4.73±0.55	0.07±0.01
Aqueous	4.40±0.20	0.07±0.01

Antioxidant activity of *M. fragrans* evaluated by DPPH **(**2, 2-diphenylpicrylhydrazyl**)** assay. In this reduction in color was observed using absorption method and calculated the IC_{50} value of various extracts.

Table 8: IC_{50} value of various extracts of *T. cordifolia* by DPPH method:

Plant Extracts	IC_{50} value of extracts	IC_{50} value of ascorbic acid
Methanol	0.26±0.20	0.07±0.01
Ethanol	3.83±0.17	0.07±0.01
Chloroform	0.49±0.03	0.07±0.01
Aqueous	4.40±0.20	0.07±0.01

IC_{50} values are calculated of free radical scavenging activity by plotting scatter graph in excel and measuring IC_{50} by slop equation. IC_{50} represents the concentration at which 50% scavenging of free radicals.

Table 9: Showing the IC_{50} of *M. fragrans* and *T. cordifolia* fractionated compounds using ascorbic acid as a standard.

Fractions of *M. fragrans*	$IC_{50\ value}$ (mg/mL)	Fractions of *T. cordifolia*	$IC_{50\ value}$ (mg/mL)
F1	8.45	TF1	--
F2	10.11	TF2	3.92
F3	9.33	TF4	0.01
F4	1.29	TF5	5.56
F5	10.44	TF6	9.85
F6	12.5	TF7	--
F7	0.11	TF8	--
F8	--	TF9	10.11
F9	--	--	--

Conclusions:-

The present study has demonstrated the antioxidative and antimicrobial activity of various extracts of *M. fragrans* and *T. cordifolia* plants and their isolated components. We have observed that methanolic extracts of both these plants have the highest antioxidant and antimicrobial properties. Antimicrobial and antioxidative properties of these plant extracts are directly related with their specific components. Our study for the first time highlights that biological activities of *M. fragrans* and *T. cordifolia* extracts are regulated by their major components. Potential of antimicrobial activity of different solvent extracts of *M. fragrans* and *T. cordifolia* against *C. sakazakii* first time evaluated. Purified compounds from both plants show significant antioxidative as well as antimicrobial activity. High antioxidant and antimicrobial activity by *M. fragrans* could be an attribute of myristicin, elimicin, eugenol and isoeugenol. Palmatine and berberine could be the possible contributor for antioxidant and antimicrobial activity of *T. cordifolia* extract. *M. fragrasns* and *T. cordifolia* seems to be promising plants regarding alternative antimicrobials against increasing numbers of pathogenic microorganisms resistant to conventional antibiotic and antioxidants which should replace the synthetic ones. However, more studies are still needed to understand and validate the mechanism of action of both plant extracts and their components. Also it is important to precede work on other extraction materials and methods because use of different solvents may be give a different compound and consequently different action. Finally, prior to use these components as antimicrobial and antioxidant agent clinical and pharmacological standardization required.

References:-

1. Albu, S., Joyce, E., Paniwnyk, L., Lorimer, J.P. and Mason, T.J. (2004): Potential for the use of ultrasound in the extraction of antioxidants from Rosmarinus officinalis for the food and pharmaceutical industry. Ultrason. Sonochem., 11: 261–265.
2. Adhvaryu, M.R., Reddy, N. and Parabia, M. H. (2007): Effects of four Indian medicinal herbs on Isoniazid-, Rifampicin- and Pyrazinamide-induced hepatic injury and immunosuppression in guinea pigs. World J Gastroenterol., 13(23): 3199–3205.
3. Albinjose, J., Jasmine, E., Selvankumar, T., Srinivasakumar K.P. (2015): Bioactive compounds of *T. cordifolia* by gas chromatography-mass spectrometry (GC-MS). Int. J. Multidiscip.. Res. Dev., 2(1): 88-97.
4. Bonvicini, F., Mandrone, M., Antognoni, F., Poli, F. and Gentilomi, G.A. (2014): Ethanolic extracts of Tinosporacordifolia and Alstoniascholaris show antimicrobial activity towards clinical isolates of methicillin-resistant and carbapenemase-producing bacteria. Nat. Prod. Res., 28(18):1438-45.
5. Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prere, M.F. and Forsythe, S.J. (2007): Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. J. Clin. Microbiol., 45(12): 3979-3985.
6. Cho, J.Y., Choi, G.J., Son, S.W., Jang, K.S., Lim, H.K., Lee, S.O., Sung, N.D., Cho, K.Y., and Kim, J.C. (2007): Isolation and antifungal activity of lignans from *Myristica fragrans* against various plant pathogenic fungi. Pest Manag. Sci., 63(9): 935–940.
7. Devi, K.P., Nisha, S.A., Sakthivel, R. and Pandian, S.K. (2010): Eugenol (an essential oil of clove) acts as an antibacterial agent against Salmonella typhi by disrupting the cellular membrane. J. Ethnopharmacol., 130(1):107-115.
8. Fasugba, O., Gardner, A., Mitchell, B.G. and Mnatzaganian, G. (2015): Ciprofloxacin resistance in community- and hospital-acquired Escherichia coli urinary tract infections: a systematic review and meta-analysis of observational studies. BMC Infect Dis., 15(1): 1282-4.

9. Gupta, A.D., Bansal, V.K. and Maithil V.B.N. (2013): Chemistry, antioxidant and antimicrobial potential of nutmeg (*Myristica fragrans* Houtt). J. Gen. Eng. Biot., 11(1): 25–31.
10. Hussain, S. P. and Rao, A. R. (1991): Chemopreventive action of mace (*Myristica fragrans*, Houtt) on methylcholanthrene-induced carcinogenesis in the uterine cervix in mice. Cancer Letters. 56(3): 231–234.
11. Hinneburg, I., Dorman, H.J.D. and Hiltunen, R. (2006): Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem., 97: 122–129.
12. Irobi, O.N., Moo-Young, M., Anderson, W.A. and Daramola, S.O. (1994): Antimicrobial activity of bark extracts of Bridelia ferruginea (Euphorbiaceae). J. Ethnopharmacol., 43: 185–190.
13. Hou, J.P., Wu, H., Wang, Y. and Weng, X.C. (2012): Isolation of some Compounds from Nutmeg and their Antioxidant Activities. Czech J. Food Sci., 30(2): 164–170.
14. Jagetia, G.C. and Rao, S.K. (2006): Evaluation of the antineoplastic activity of guduchi (*Tinospora cordifolia*) in Ehrlich ascites carcinoma bearing mice," Biological and Pharmaceutical Bulletin., 29(3): 460–466.
15. Jayaprakash, R., Ramesh, V., Sridhar, M.P. and Sasikala, C. (2015): Antioxidant activity of ethanolic extract of *Tinospora cordifolia* on N nitrosodiethylamine (diethylnitrosamine) induced liver cancer in male Wister albino rats. J. Pharm Bioallied Sci., 7(1): 40–45.
16. Kala, C.P., Dhyani, P.P. and Sajwan, B.S. (2006): Developing the medicinal plants sector in northern India: challenges and opportunities. J. Ethnobiol Ethnomed., 2: 32.
17. Kim, Y.M., Ha, Y.M., Jin, Y.C., Shi, L.Y., Lee, Y.S., Kim, H.J., Seo, H.G., Choi, J.S., Kim, Y.S., Kang, S.S., Lee, J.H. and Chang, K.C. (2009): Palmatine from Coptidisrhizoma reduces ischemia-reperfusion-mediated acute myocardial injury in the rat. Food Chem Toxicol., 47(8): 2097–2102.
18. Lambert, R.J.W., Skandamis, P.N., Coote, P.J. and Nychas, G.J.E. (2001): A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J. App. Microbio., 91(3): 453–462.
19. Lai, K.K. (2001): *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. Medicine., 80: 113–122.
20. Lim, J.Y., Yoon, J.W. and Hovde, C.J. (2010): A Brief Overview of *Escherichia coli* O157:H7 and Its Plasmid O157. J Microbiol Biotechnol., 20(1): 5-14.
21. Mishra, M.P., Rath, S., Swain, S.S., Gosh, G., Das, D. and Padhy R.N. (2015): *In vitro* antibacterial activity of crude extract of 9 selected medicinal plants against UTI causing MDR bacteria. J King Saud Univ Sci.
22. More, P. and Pai, K. (2012): *In vitro* NADH-oxidase, NADPH-oxidase and myeloperoxidase activity of macrophages after *Tinospora cordifolia* (guduchi) treatment. Immunopharmacol Immunotoxicol., 34:368–372.
23. Nakai, M., Harada, M., Akimoto, K., Shibata, H., Miki, W. and Kiso, Y. (2003): Novel antioxidant metabolites in Rat liver with ingested Sesamin. J. Agric. Food Chem., 51(6): 1666–1670.
24. Narasimhan, B. and Dhake, A.S. (2006): Antibacterial principles from *Myristica fragrans* seeds. J. Med. Food., 9(3): 395–399.
25. Njume, C., Afolayan, A.J., Green, E. and Ndip, R.N. (2011): Volatile compounds in the stem bark of Sclerocarya birrea possess antimicrobial activity against drug resistant strains of Helicobacter pylori. Int. J. Antimicrob. Agents., 38(4): 319–324.
26. Ozaki, Y., Soedigdo, S., Wattimena, Y.R. and Suganda, A.G. (1989): Antiinflammatory effect of Mace, aril of *Myristica fragrans* HOUTT., and its active principles. JPN J Pharmacol., 49(2): 155–163.
27. Omoruyi, I.M. and Emefo, O.T. (2012): *In Vitro* evaluation of the antibiogramic activities of the seeds of *Myristica fragrans* on food borne pathogens. Malays J Microbiol., 8(4): 253-258.
28. Pandey, R., Rameshkumar, K.B. and Kumar, B. (2015): Ultra high performance liquid chromatography tandem mass spectrometry method for the simultaneous determination of multiple bioactive constituents in fruit extracts of *Myristica fragrans* and its marketed polyherbal formulations using a polarity switching technique. J Sep Sci., 38(8):1277-85.
29. Patel, M.B. and Mishra, S. (2011): Hypoglycemic activity of alkaloidal fraction of *Tinospora cordifolia*. Phytomedicine., 18(12):1045–52.
30. Pandey, A. K., Mishra, A. K. and Mishra, A. (2012): Antifungal and antioxidative potential of oil and extracts derived from leaves of indian spice plant Cinnamomum tamala. Cell Mol Biol., 58(1): 142–147.
31. Qiu, Q., Zhang, G., Sun, X. and Liu, X. (2004): Study on chemical constituents of the essential oil from *Myristica fragrans* Houtt. by supercritical fluid extraction and steam distillation. Journal Chinese Medicinal Materials., 27(11): 823–826.
32. Raccach, M. (1984): The antimicrobial acticity of phenolic antioxidants in food—a review," J. Food Safety., 6(3): 141–170.
33. Shan, B., Cai, Y.Z., Sun, M. and Corke, H. (2005): Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J. Agric. Food Chem., 53(20): 7749–7759.
34. Sharma, U., Bala, M., Kumar, N., Singh, B., Munshi, R.K. and Bhalerao, S. (2012): Immunomodulatory active compounds from *Tinospora cordifolia*. J Ethnopharmacol., 141(3): 918–26.

35. Shafiei, Z., Shuhairi, N.N., Shah yap, N.M.F., Sibungkil, C.H. and Latip, J. (2012): Antibacterial Activity of *Myristica fragrans* against Oral Pathogens. J. Evid. Based Complementary Altern., Med. 2012(2012): 7.
36. Singh, S and Singh, P. (2012): Effectiveness of *T. cordifolia* stem extract on bacteria Salmonella typhi, Pseudomonas aeruginosa, staphylococcus aureus and Shieglla dysenteriae. Int. J. Pharma life sci., 3(8): 1923-1925.
37. Sazia, M., Saba, M., Jasmine, F., Farhan, K. and Huma, M. (2015): Antibacterial, antioxidant and cytotoxic potential of various extracts of *Myristica fragrans*. Int. J. Ayur Pharm., 6(5): 643-648.
38. Sonavane, G.S., Sarveiya, V.P., Kasture, V.S. and Kasture, S.B. (2002): Anxiogenic activity of *Myristica fragrans* seeds. Pharmacol. Biochem. Behav., 71(1-2): 239–244.
39. Singh, S.S., Pandey, S.C., Srivastava, S., Gupta, V.S., Patro, B. and Ghosh, A.C. (2003): Chemistry and medicinal properties of *Tinospora cordifolia* (Guduchi). Indian J Pharmacol., 35(2): 83–91.
40. Tajuddin., Ahmad, S., Latif, A. and Qasmi, I.A. (2003): Aphrodisiac activity of 50% ethanolic extracts of *Myristica fragrans* Houtt. (nutmeg) and *Syzygium aromaticum* (L) Merr. & Perry. (clove) in male mice: a comparative study. BMC Complement. Altern. Med., 3: 6–10.
41. Torbati, M., Nazemiyeh, H., Lotfipour, F., Nemati, M., Asnaashari, S. and Fathiazad, F. (2014): Chemical composition and in vitro antioxidant and antibacterial activity of*Heracleum transcaucasicum* and *Heracleum anisactis* roots essential oil. Bioimpacts., 4(2): 69-74.
42. Takikawa, A., Abe, K., Yamamoto, M., Ishimaru, S., Yasui, M., Okubo, Y. and Yokoigawa, K. (2002): Antimicrobial activity of nutmeg against Escherichia coli O157. J. Biosci. Bioeng., 94(4): 315–320.
43. Yano, Y., Satomi, M. and Oikawa, H. (2006): Antimicrobial effect of spices and herbs on Vibrio parahaemolyticus. Int. J. Food Microbiol., 111(1): 6–11.
44. Yoon, S.S., Hennigan, R.F., Hilliard, G.M., Ochsner, U.A., Parvatiyar, K., Kamani, M.C., Allen, H.L., DeKievit, T.R., Gardner, P.R., Schwab, U., Rowe, J.J., Iglewski, B.H., McDermott, T.R., Mason, R.P., Wozniak, D.J., Hancock, R.E., Parsek, M.R., Noah, T.L., Boucher, R.C. and Hassett, D.J. (2002): Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev cell., 3(4):593-603.
45. Zaidi, S.F.H., Yamada, K., Kadowaki, M., Usmanghani, K. and Sugiyama, T. (2009): Bactericidal activity of medicinal plants, employed for the treatment of gastrointestinal ailments, against Helicobacter pylori. J. Ethnopharmacol., 121(2): 286–291.