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Strong Sour Tamarind Flavor of Methyl-2,3,4-trihydroxyhexanoate, a new compound isolated from Leaves of *Tamarindus indica*, L. plays a role in plant defense mechanisms

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ABSTRACT

Flavoring compounds of plants play a significant role in plant defense mechanism. Compound responsible for strong sour tamarind flavor has been isolated and identified from Methanol fraction of tamarind leaves (TrMF). Chromatographic and spectral analyses of TrMF revealed the compound to be methyl 2,3,4-trihydroxyhexanoate. This compound showed a strong antioxidant activity as well as strong antimicrobial activity. It showed significant antioxidant activity with Ic50 value of 2.5µg/ml whereas tert-butyl-1-hydroxytoluene and ascorbic acid revealed 26.0µg/ml and 5.0µg/ml, respectively. It also revealed strong inhibitory activity against Aspergellosis disease-causing fungi namely; *Aspergillus fumigatus, Aspergillus tamarii* and *Aspergillus niger* at all concentrations. *Streptococcus aureus* and *Escherichia coli* were much more sensitive to methyl-trihydroxy-hexanoate at all concentrations than *Pseudomonas aeruginosa*. This pure compound exhibited concentration dependent inhibitory and stimulatory activity on rice seeds germination and seedling growth. It showed strong inhibitory activity up to 62.5ppm concentration and below this concentration the effect was stimulatory. Methyl- trihydroxyhexanoate exhibited wide range of defensive activity against microbes and crop seeds and also possesses potent antioxidative activity. Thus play an important role in plant defense mechanism and can be utilized as a valuable source of bio-herbicides and pesticides.

Key Words: Tamarind (*Tamarindus indica* L.), Tamarind flavor, Tamarind methanol fraction, Methyl 2,3,4-trihydroxyhexanoate, Biopesticide.

Abbreviations: Tamarind methanolic fraction (TrMF), Methyl 2,3,4-trihydroxyhexanoate (MTHH), Dimethyl Sulfoxide (DMSO).

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INTRODUCTION

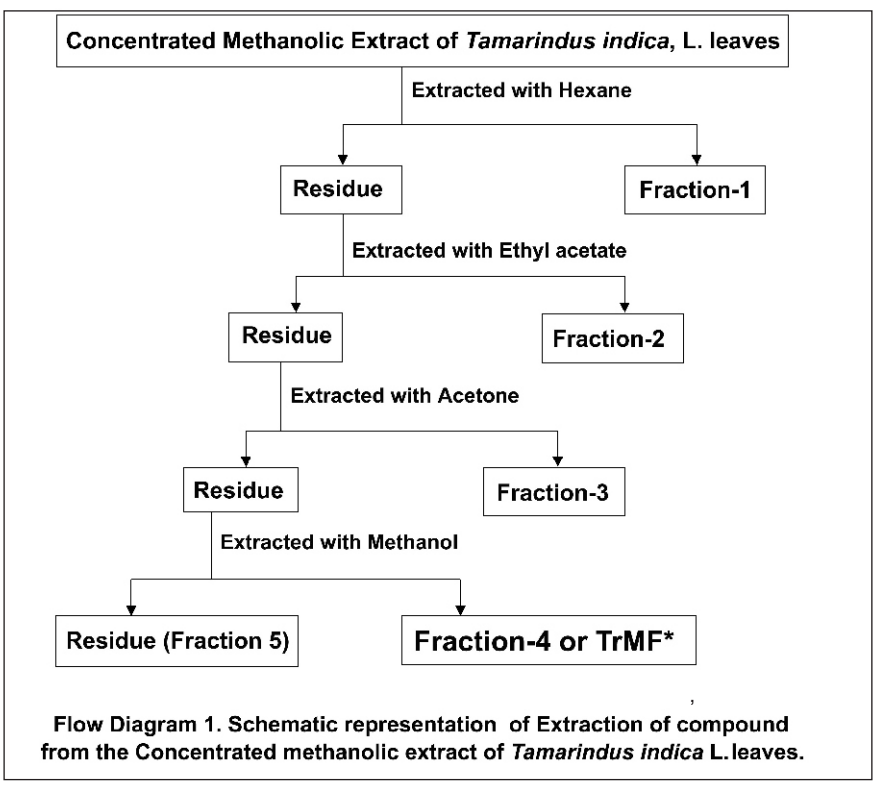
Allelochemicals are plant metabolites released into the environment through volatilization; exudation from roots, leaching from plants or plant residues, and decomposition of residues (Waller, 1987; Putnam and Tang, 1986; Einhellig, 1995; Halbrendt, 1996). The use of allelopathic compounds either in their native or processed forms for the management of plant diseases is an important approach. This approach is receiving increased attention as highly toxic chemical biocides are discouraged. In general, these compounds are less toxic to non-target species and less persistent in soil. Allelochemicals are sometimes produced in large quantities in plant material or as agricultural waste, making the use of rotation crops, cover crops, and organic amendments as effective means for production and/or distribution of the active compounds (Anaya *et al*,1990). Identified strong bioactive allelochemicals are a useful source for the development of biological herbicides and fungicides (Inderjit and Mukherji, 2006). The current trend in agriculture is to find a biological solution to reduce the perceived hazardous impacts of herbicides and insecticides (Khanh *et al*, 2005). The present report indicates a way of producing natural biopesticides, which can replace commercial pesticides.

Tamarind trees shed leaves in all seasons and vegetation under the tree is very sparse. In the early stages, Tamarind seedlings release inhibitors through their roots and in mature stage they release inhibitors through leaves also. Tamarind leaves have very toxic allelopathic effects (Mandal and Tapaswi, 1997). Later Parvez *et al*, (2003; 2004) studied the allelopathic activity of Tamarind leaves, bark and seed. In our earlier work we have isolated putative caffeic acid derivative with strong antimicrobial activity from the ethyl acetate fraction of Tamarind leaves (Biswas et al, 2009). In the present study, we are interested in isolating and studying the bioactivity of the compound responsible for strong sour tamarind flavor from leaves of *Tamarindus indica* L because flavoring agents of plants play a significant role in plant defense mechanism.

MATERIALS AND METHODS

Isolation and Characterization of Active Allelochemical

Tamarind (*Tamarindus indica* L.) leaves (200gm) were dried and powdered using Sample Miller Machine (Cyclotec 1093. Sample Mill, TECATOR) and then soaked in 500ml of methanol for 7 to 10 days. The entire mixture was then vortexed in high speed (3000 rpm) using Mechanical Stirrer (Model No. DC Stirrer NZ-1000s AC220V, EYELA) for 2hr and then filtered through sintered disc funnel. The brown colored extract was collected and concentrated in a rotary vacuum evaporator (EYELA, Model No. N1-NW) and subsequently extracted with hexane, ethyl acetate, acetone and methanol, respectively (Flow Diagram 1). Finally the compounds were purified by column chromatography and thin layer chromatography. Here we emphasis on Methanol fraction of Tamarind (henceforth referred to as TrMF) for biological activity



Flow Diagram 1. Schematic representation of Extraction of compound from the Concentrated methanolic extract of *Tamarindus indica* L. leaves.

Thin Layer Chromatography (TLC)

TLC plates (20x20 cm) were used for this study. Silica gel G of TLC grade was used as a coating material and plates were coated uniformly with 0.5mm thick layer of silica gel. A solvent mixture in the ratio of 9.5: 0.5: Acetone: Methanol was taken as solvent system (Stahl, 1969). The polar solvents were mostly preferred for the extraction process because the fraction we worked upon was methanolic and moreover the compound was found to be readily soluble in polar solvents than those of 20µl solvents. Plates were loaded with 20µl solution (500ppm of TrMF) and developed up to a height of 18 cm in glass chamber pre-saturated with desired solvent system. TLC plated were then taken out and dried under a stream of hot air. Finally compounds were detected by exposing the plates under iodine vapor.

Spectral Analysis

LC-MS Analysis for determination of molecular weight of the purified TrMF of *Tamarindus indica* was done with Mass Spectrometer (Micromass Q-TOF Micro™ available at IICB, Jadavpur, Kolkata 700 032) in its positive ion mode

^{1}HNMR Analysis of purified TrMF was performed with the help of 600 MHz NMR Spectrometer (PROBHD 5mm DUEL 13C1, PULPROG Zg3D, TD32768, SOLVENT $CDCl_3$) available at Chembiotek, Kolkata 700054. ^{1}HNMR spectra were detected on δ ppm (0-10) scale with end sweep at 0 ppm. ^{13}CNMR Analysis was also recorded with the help of 600 MHz NMR Spectrometer (PROBHD 5mm DUEL 13C1, PULPROG zgpg, TD65536, SOLVENT $CDCl_3$) available at Chembiotek, Kolkata 700054. ^{13}CNMR spectra of purified TrMF on δ ppm (0-200) scale with end sweep at 0ppm. In both the cases samples were analyzed at ambient temperature and $CDCl_3$ was used as solvent for dissolving the compound.

FTIR analysis was conducted to confirm the important functional group in the extracted and purified TrMF of *Tamarindus indica* with the help of FTIR Spectrometer (Model No.QC/FTIR/006 available at Chembiotek, Kolkata 700054).

Antioxidant Activity of TrMF of Tamarindus Indica

The antioxidant or free radical scavenging activity of the extracts [i.e. Hexane fraction (TrHF), ethyl acetate fraction (TrEtoAcF), Acetone Fraction (TrAF) and methanol fractions (TrMF)] of Tamarind leaves (Ravishankara et al., 2002) on the stable radicle 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Brand-Willims et al (Brand-Willims et al.,1995). In the experiment, 2.0mg of pure TrMF compound was dissolved in methanol. Solution of varying concentrations such as 500µg/ml, 250µg/ml, 125µg/ml, 62.50µg/ml, 31.25µg/ml, 15.62µg/ml, 7.8125µg/ml, 3.91µg/ml, 1.95µg/ml and 0.98µg/ml were obtained from serial dilution technique. 2ml of the methanol solution of TrMF of each concentration was mixed with 3ml of a DPPH-methanol solution (20µg/ml) and was allowed to stand for 20 minutes for the reaction to occur. Then the absorbance was determined at 517nm and from these values the corresponding percentage of inhibition were calculated by using the following equation:

$$\% \text{ inhibition} = [1-(ABS_{sample} / ABS_{control})] \times 100.$$

Then percentage inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated by using tert-butyl-1-hydroxytoluene (BHT), ascorbic acid, potential antioxidant, were used as positive control.

Effects of Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at Different Concentrations on Fungi

Inhibition zone test technique was performed for testing the impact of Methyl-2,3,4-trihydroxyhexanoate against three different fungal species viz. *Aspergillus niger, Aspergillus fumigatus* and *Aspergillius tamarii.* Few fungal spores of test fungi were transferred to PDA (Potato Dextrose Agar Media) slants and incubated for one week for colony growth. After one week, one loop full of fungal spore of each species was added separately to the sterile saline water and mixed well. Fungal spore suspension (1 ml) in water was then poured in a sterile Petri dish containing molten PDA and allowed to solidify the plates. A stock solution of 2000ppm was prepared by dissolving 30mg of Methyl-2,3,4-trihydroxyhexanoate in 15ml of DMSO (Dimethyl Sulfoxide). Serial dilutions with decreasing stock volume and increasing solvent volume was made for 4ml of each concentration. 3ml of stock was dissolved with 1ml of the DMSO to make

up 1500ppm, 2ml of stock with 2ml of DMSO to make up 1000ppm and finally 1ml of stock with 3ml of DMSO to make up 500ppm. Four cups (25mm^2 size) were cut at equidistant positions and in these cups 0.5 ml solution of Methyl-2,3,4-trihydroxyhexanoate at 500ppm, 1000ppm, 1500ppm, and 2000ppm was added. Treated plates were incubated at 28±1°C for 24-48 hrs. After 48 hrs plates were taken out and observations were recorded for colony growth inhibition. Area of inhibition zone was calculated as: Area of inhibition at x ppm = 3.14 ($TR_x^2 - r^2$) where x = concentration used; r = radius, TR = Total radius of the inhibition zone at specific concentration (Biswas et al, 2009).

Effects of Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at Different Concentrations on Bacteria

Effects of Methyl-2,3,4-trihydroxyhexanoate against three different bacterial species viz. *Escherichia coli* and *Pseudomonas aeruginosa* were measured by using inhibition zone test. Firstly the strains of test bacteria were transferred to NA (Nutrient Agar Media) slants and incubated for 24 hrs. After 24 hrs., 1 loop full of bacterial spore of each test species was added separately to sterile nutrient broth and mixed well and incubated at 37°C for two and a half hour. Bacterial spore suspension (1 ml) in sterile nutrient broth was then added to sterile Petri dish containing molten NA medium and allowed to solidify. After complete solidification four cups (25 mm^2 size) were cut at equidistant positions and in these cups 0.5 ml solution of Methyl-2,3,4-trihydroxyhexanoate at 500ppm, 1000ppm, 1500ppm, and 2000ppm were prepared as mentioned above and was added. Treated plates were incubated at 37±1°C for 24 hrs. After this period, plates were taken out and observations of inhibition zone were recorded, using the formula described for fungal effects.

Effects of Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at Different Concentrations on the Germination and Subsequent Growth of Rice

The allelopathic potentials of isolated and purified Methyl-2,3,4-trihydroxyhexanoate on the seedling growth of rice as well as the effect on germination of rice were determined by bioassay experiments. 30 mg of Methyl-2,3,4-trihydroxyhexanoate was dissolved in 30ml of distilled water. This constituted the stock solution of 1000ppm from which further dilutions 500, 250, 125, 62.5, 31.25, 15.62, 7.81ppm were made. In one experiment nine sets of petridish bioassay including control were performed.

This experiment was replicated three times. In the control set 15ml of distilled water was added instead of test solution. Seeds were surface sterilized with 0.1% mercuric chloride solution, washed with distilled water and placed on a filter paper in petridish. After 4 days, shoot length and root length in the control and treated sets were measured (Mandal, 2001).

RESULTS

Isolation and Characterization of Active Allelochemical

Thin Layer Chromatography Analysis

After purification TrMF (methanol fraction of tamarind leaves) was run on TLC in the solvent system (9.5: 0.5 :: Acetone : Methanol) showed single bright yellow spot in iodine vapor with Rf value 0.81. Purified TrMF was brown in color with strong sour tamarind smell and physical state of compound was oily at room temperature.

Spectral Analysis

Mass spectra of purified TrMF (Fig. 1) showed base peak at m/z 201.03 and another small peak at 179.04 corresponding to $[M+Na]^+$ and $[M+H]^+$ respectively. Hence molecular weight of the compound is (201-23) 178.

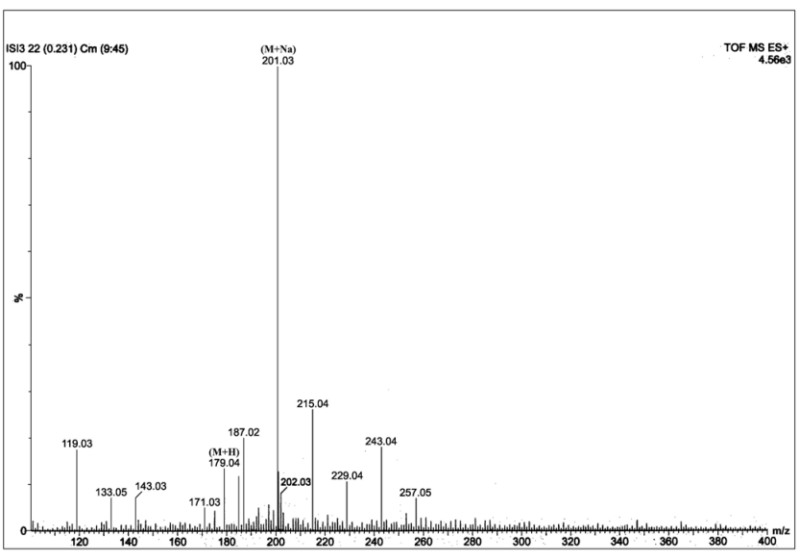


Fig.1. LCMS Spectra of TrMF of *Tamarindus indica* L.

From the ^{1}HNMR spectra of purified compound of TrMF (Fig.2a and 2b), it is clear that the methyl proton of the ester group is highly deshielded due to the presence of electronegative oxygen atom and appeared in the region ~δ 3.8. The terminal -CH_3 proton appeared in the most shielded region ~ δ 1.3 value and the proton was expected to produce multiplet with viscinal coupling with the - CH_2 group and geminal coupling with itself. The -CH_2 proton α with respect to the CO group was expected to produce a most deshielded environment due to the presence of –I effect of two –OH group and a Carbonyl moiety. That is why these protons come in the region of ~δ 4.5 with triplet. This splitting is expected to be a good one due to the pronounced effect of the deshielding zone of the carbonyl group. The -CH_2 proton β with respect to the CO group appeared at ~δ 4.1 with a multiplet with two sets of viscinal (-OH) coupling as well other -CH_2 group in the chain differ little in their chemical shift as the presence of +I effect of right hand ethyl group and appeared in the little shielded region of ~δ 3.54. The presence of –OH protons are evident by three bands at ~δ 2.1 region due to vicinal coupling with neighboring –CHOH groups.

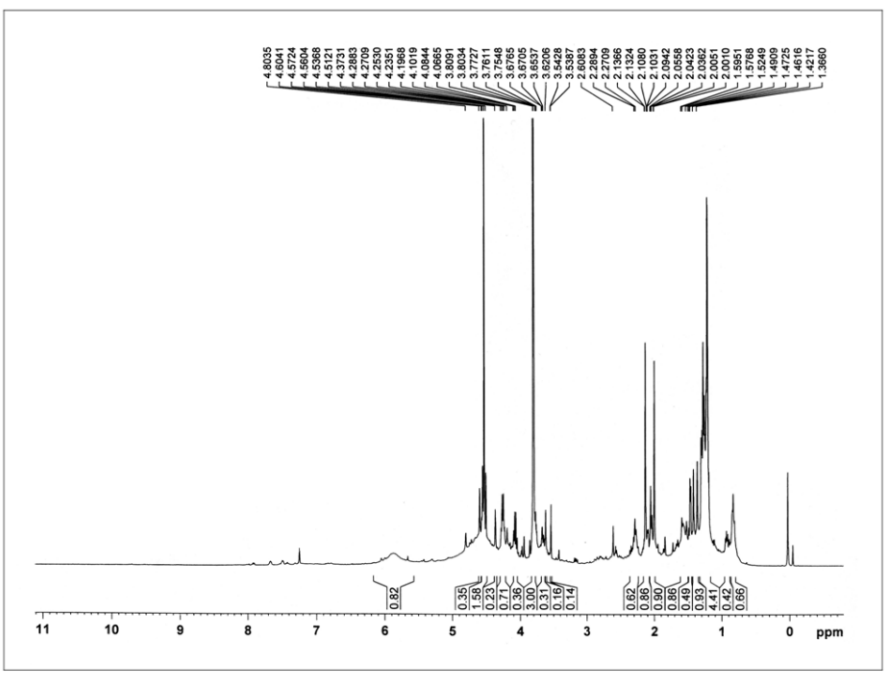


Fig.2a.^{1}HNMR Spectra of Purified TrMF of *Tamarindus indica*, L.

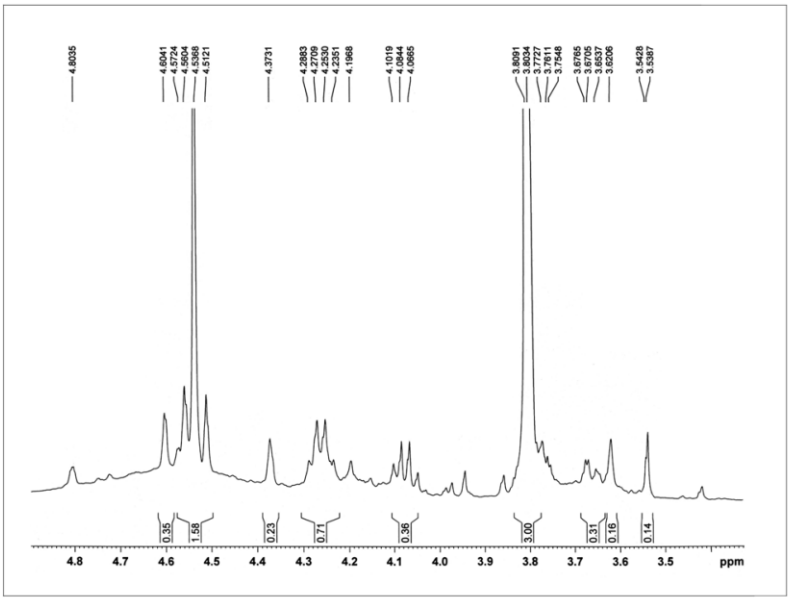


Fig.2b.Expanded ^{1}HNMR Spectra of Purified TrMF of *Tamarindus indica*, L.

Fig 3a, 3b and 3c showed ^{13}CNMR spectra of purified TrMF of *Tamarindus indica*. One small peak at δ 171ppm definitely pertained to the carbonyl carbon of ester group. The triplet signal at δ (77.693-77.185ppm) appeared for the $CDCl_3$ (solvent used for dissolving the compound). The –O-CH_3 carbon signal occurred at δ 52.9ppm due to the deshielding by electronegative oxygen atom. The ^{13}C signal of the α carbon to the carbonyl group appeared at δ 71.8ppm. The terminal CH_3 signal appeared in the shielded region of δ 14.05ppm. The –CH_2 carbon signals appeared at in the range of δ (26ppm) region. Therefore ^{13}CNMR analysis also suggested to be a short chain saturated ester. Also the structure was further confirmed by DEPT-135 experiment as the quaternary CO signal of ester was vanished and all the signal of - CH_2 group was inverted but two - CH_3 signals remained unchanged.

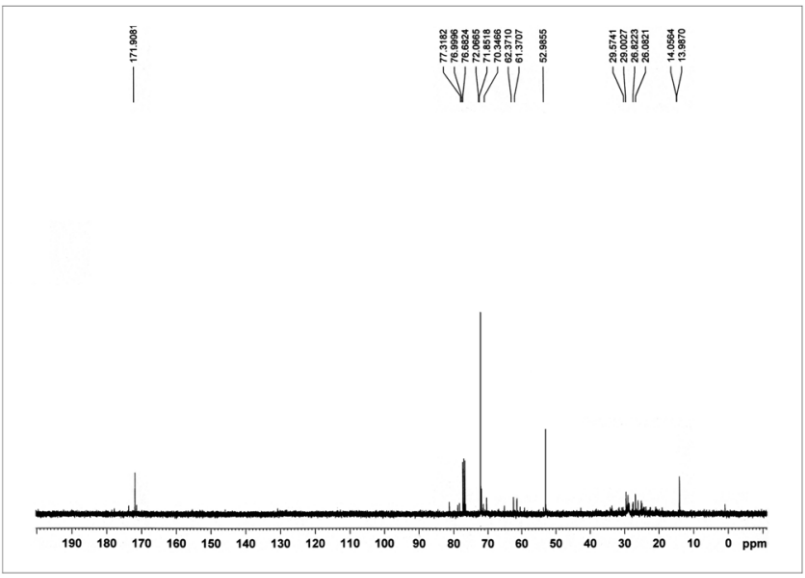


Fig.3a.^{13}CNMR Spectra of Purified TrMF of *Tamarindus indica*, L.

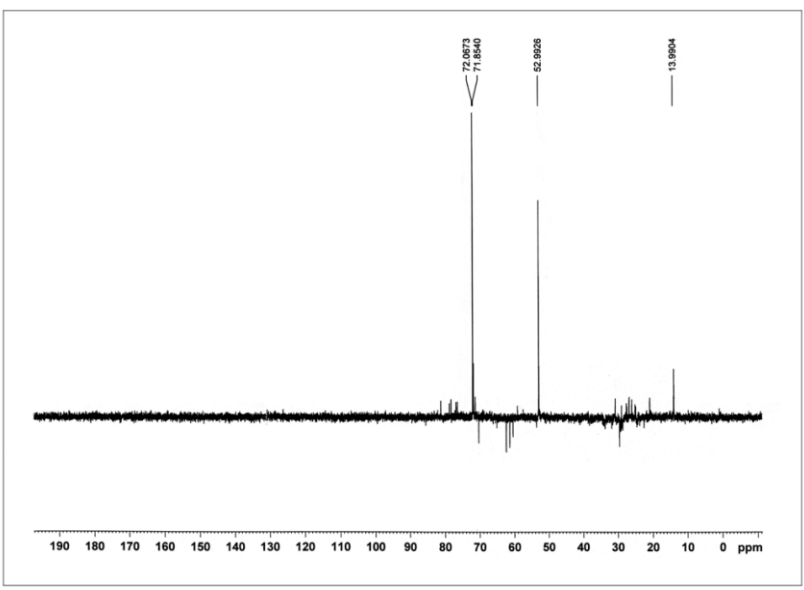


Fig.3b. Dept-135 Spectra of Purified TrMF of *Tamarindus indica*, L.

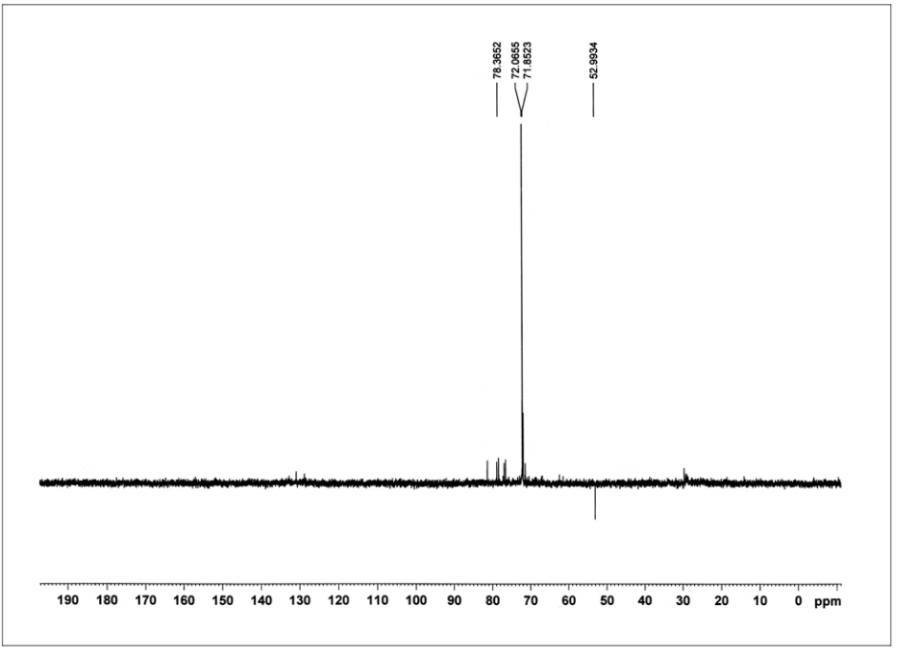


Fig.3c. Dept-90 Spectra of Purified TrMF of *Tamarindus indica*, L.

IR Spectra of purified TrMF showed five characteristic peaks (Fig.4). Among them, peak at 2928 cm^{-1} and 2857 cm^{-1} was probably for the asymmetrical (ν_{as} CH_2) and symmetrical stretching (ν_s CH_2) of methylene group, respectively (Silverstein and Webster,1997). Peak at 1377 cm^{-1} indicated the symmetrical bending vibration (δ_S CH_3) of the methyl group and peak at 1444 cm^{-1} suggested the asymmetrical bending vibration (δ_{as} CH_3) of the methyl group. Peak at 1742 cm^{-1} confirmed the C=O absorption band of saturated aliphatic esters. A broader peak at 3444 cm^{-1} strongly indicate the presence of hydroxyl moiety as well as strong hydroxyl hydrogen bonding between them. So it can be suggested that the compound is a saturated one and contained only the ester group and hydroxyl group as the functional group.

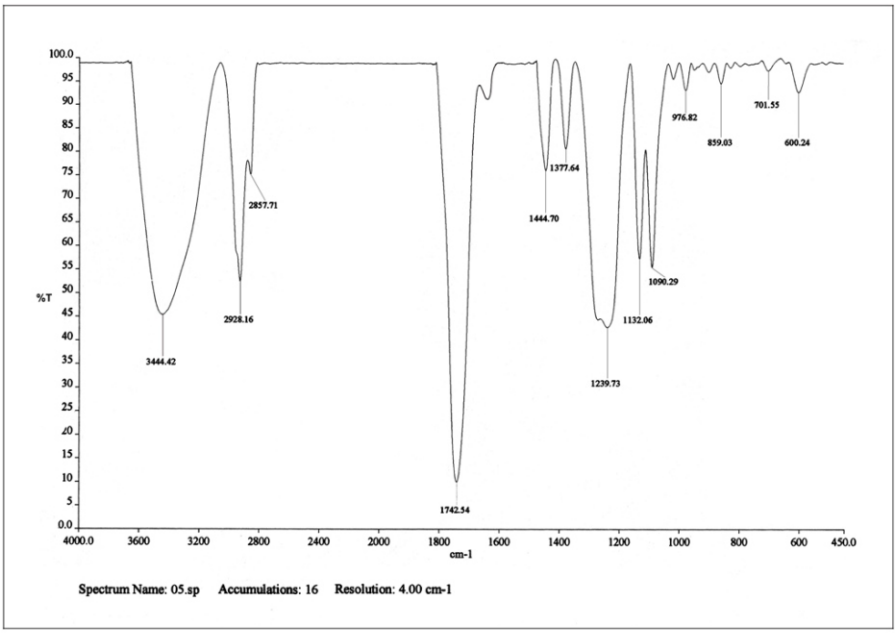


Fig.4. FTIR Spectra of Purified TrMF of *Tamarindus indica*, L.

Chromatographic and spectral analyses of TrMF revealed the compound to be methyl 2,3,4-trihydroxy-hexanoate (Fig.5). In fact, there are no reports on the presence of Methyl-2,3,4-trihydroxyhexanoate in *Tamarindus indica* leaves.

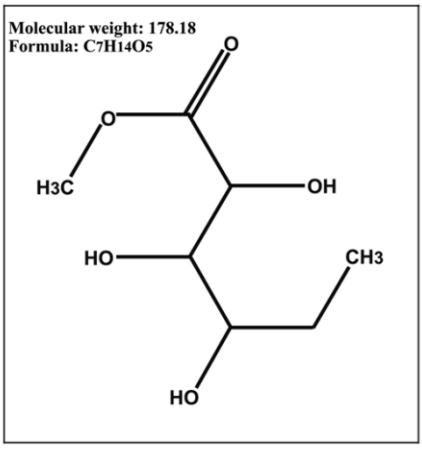


Fig. 5: Molecular structure of Methyl 2,3,4-trihydroxy-hexanoate isolated from *Tamarindus indica* L.

Antioxidant Activity of TrMF of Tamarindus Indica

In case of antioxidant screening (Table 1), the IC_{50} value of BHT and ascorbic acid (AS) obtained was 26.0µg/ml and 5.0µg/ml respectively. In this investigation, the methanol extract (TrMF) of the plant showed the highest antioxidant activity with IC_{50} value of 2.5 µg/ml. Acetone soluble fraction (TrAF) of the methanol extract also revealed potent antioxidant activity (IC_{50}=4.0µg/ml). On the other hand the ethyl acetate (TrEtoAcF) and n-hexane soluble fraction (TrHF) showed moderate antioxidant activity. So we find the Methanol Fraction of *Tamarindus indica* with the maximum antioxidant activity.

Table 1. IC_{50} values of different fractions of *Tamarindus indica,* L. along with tert-butyl-1-hydroxytoluene and ascorbic acid.

Samples	IC_{50} (µg/ml)
BHT	26.0
AS	5.0
TrHF	7.0
TrEtoAcF	5.5
TrAF	4.0
*TrMF	*2.5

Effects of Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at Different Concentrations on Fungi

In the inhibition zone test, three fungal species viz. *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillius tamarii* showed different diameters of inhibition zone at different concentrations of Methyl-2,3,4-trihydroxyhexanoate (Fig.6). *A. fumigatus, A. niger* and *A. tamarii* are very sensitive to Methyl-2,3,4-trihydroxyhexanoate at all concentrations. In case of *A. fumigatus,* maximum inhibitory activity was noticed at 1000ppm whereas in *A. niger* and *A. tamarii,* 1500ppm exerted maximum inhibitory effects.

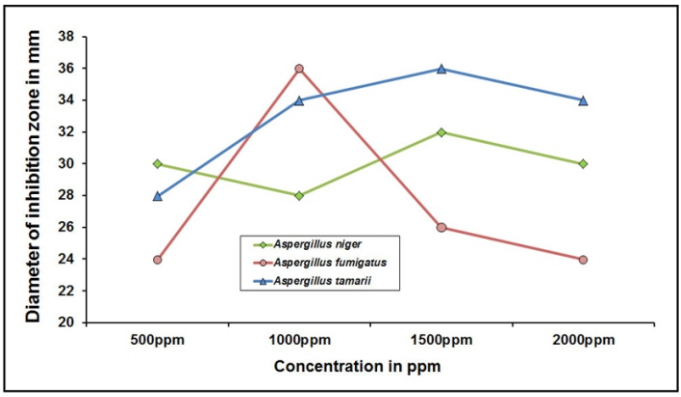


Fig.6: Effects of TrMF of *Tamarindus indica*, L. at different concentrations on *Aspergillus niger, A. Fumigatus and A. tamarii.* Correlation is significant at 0.01 levels (Pearson 2-tailed).

Effects of Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at Different Concentrations on bacteria

Escherichia coli and *Streptococcus aureus* were more sensitive to Methyl-2,3,4-trihydroxyhexanoate at all concentrations than *Pseudomonas aeruginosa*. In case of *P. aeruginosa*, 500ppm and 2000ppm showed higher inhibitory effect than lower concentration used (Fig 7). In *E. coli* and *S. aureus,* higher inhibitory effects were detected at 500ppm and 1000ppm respectively. These differential inhibitory activities are due to the dose-dependent activity of the compound.

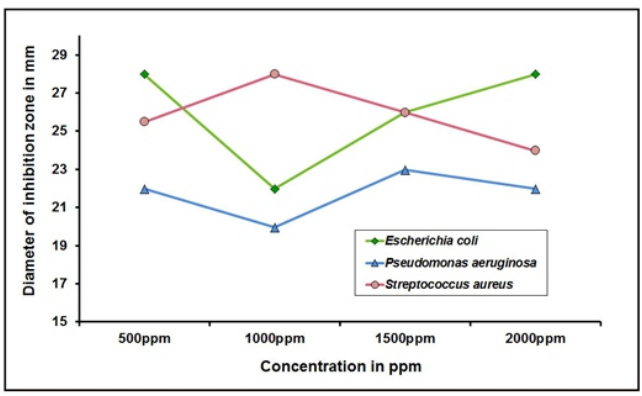


Fig.7. Effects of TrMF of *Tamarindus indica* at different concentrations on *Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus aureus.* Correlation is significant at 0.01 levels (2-tailed).

Bioassay with Methyl-2,3,4-trihydroxyhexanoate of Tamarindus indica at different concentrations on the germination and subsequent growth of rice

Methyl-2,3,4-trihydroxyhexanoate showed concentration dependent inhibitory and stimulatory activity on rice seeds (var. Shamali). It showed complete inhibition on germination from 1000ppm to 250ppm concentrations. At 125ppm it revealed 93.19% inhibition on shoot length and 97.00% inhibition on root length. At 62.5ppm concentration, 37.78% and 43.09% inhibition on shoot length and root length were noticed respectively. Below this concentration effects are stimulatory for both shoot and root length. At 31.25ppm concentration. it revealed 1.26% stimulation on shoot length and 17.47% stimulation on root length. At 15.62ppm concentration, 17.58% and 33.44% inhibition on shoot length and on root length were recorded respectively. At 7.81ppm concentration., 24.63% stimulation on shoot length and 36.77% stimulation on root length were detected (Fig.8).

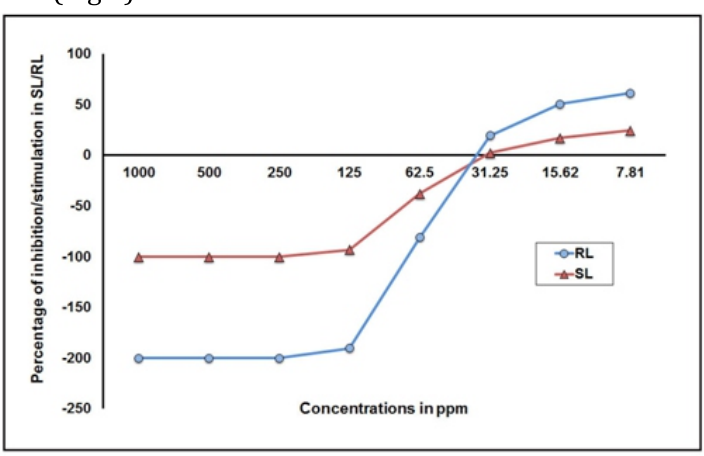


Fig.8. Effects of TrMF of *Tamarindus indica*, L. at different concentrations on germination and seedling growth of rice. SL and RL indicates shoot and root length respectively. Correlation is significant at 0.05 levels (Pearson 2-tailed).

DISCUSSION

Plants produce a huge array of natural products (secondary metabolites). These compounds have important ecological functions, providing protection against attack by herbivores and microbes and serving as attractants for pollinators and seed-dispersing agents. They may also contribute to competition and invasiveness by suppressing the growth of neighboring plant species (allelopathic interaction). Humans exploit natural products as sources of drugs, flavoring agents, and fragrances and for a wide range

of other applications. Secondary metabolites are commercially important as medicinal substances, fragrances, food additives (pigments, flavoring and aromatic compounds) and pesticides (Heble et al, 1983; Kurz,1989).

Compound responsible for strong sour tamarind flavor has been isolated and identified from Methanol fraction of tamarind leaves (TrMF). Chromatographic and spectral analyses of TrMF revealed the compound to be methyl 2,3,4-trihydroxyhexanoate. There are no reports on the presence of methyl 2,3,4-trihydroxyhexanoate in Tamarind Leaves. This active ingredient showed antifungal activity on *Aspergillus fumigatus, Aspergillus niger*, *Aspergillus tamarii* and all the three species were highly sensitive to Methyl 2,3,4-trihydroxyhexanoate at all concentrations (500ppm, 1000ppm, 1500ppm and 2000ppm). It also exhibited antibacterial activity on *Escherichia coli, Streptococcus sp. and Pseudomonas aeruginosa. E. coli* and *S. aureus* were much more sensitive to TrMF allelochemicals at all concentrations but in *P. aeruginosa.* 500ppm and 2000ppm showed higher inhibitory effect than 1000ppm and 1500ppm. The TrMF fraction of *Tamarindus indica* also exhibited a strong anti-oxidant property (IC_{50} value 2.5 µg/ml) which enables tamarind to serve as excellent preservatives. This pure compound showed concentration dependent inhibitory and stimulatory activity on rice seeds. It showed complete inhibition on germination from 1000ppm to 250ppm concentrations. At 125ppm it revealed 93.19% inhibition on shoot length and 97.00% inhibition on root length. At 62.5ppm concentration, 37.78% and 43.09% inhibition on shoot length and root length was noticed respectively. At 7.81ppm conc., 24.63% stimulation on shoot length and 36.77% stimulation on root length were detected. This pure compound exhibited wide range of defensive activity against microbes and crop seeds and may be utilized as a bioactive herbicide as well as pesticide.

The knowledge and use of plants as flavoring and seasoning to enhance the quality of foods, beverages and drugs is as old as the history of mankind. In future, the commercialization of this bioactive ingredient may be utilized as a plant defense compound in Agricultural practices and in other crop-enhancement strategies.

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