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Allelochemicals from the leaves of *Tamarindus indica* L.

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ABSTRACT

Potent biopesticides with strong antimicrobial activity as well growth inhibitory activity has been isolated and purified from the Ethyl Acetate Fraction (TrEAF) of tamarind leaves. Chromatographic analyses (TLC, UV, MS, NMR, and IR) indicated the presence of putative caffeic acid derivative in Tamarind leaves. *Aspergillus tamarii* was highly sensitive to TrEAF compound at all concentrations (500 ppm, 1000 ppm, 1500 ppm and 2000 ppm) than *Aspergillus fumigatus*, *Aspergillius niger*. In *Aspergillus tamarii* and *Aspergillius niger*, 1500ppm concentration was very much sensitive to TrEAF compound. *Cercina lutia*, *Staphylococcus aureus*, *Micrococcus roseus* and *Pseudomonas* sp were much more sensitive to TrEAF compound than *Escherichia coli*. No effect of TrEAF compound was detected in *E.coli* at concentration of 1000ppm. When isolated and purified by fractionation and column chromatography, this active TrEAF compound showed concentration-dependent inhibitory/stimulatory activity on rice seeds. At concentration of 1000ppm, it showed 53.81% inhibition on shoot length and 58.85% inhibition in root length. At 500ppm concentration, 29.01% inhibition in shoot length and 38.82% inhibition in root length have been detected. Very slight stimulation on shoot length was observed at 7.81ppm. It showed no effect on the germination of rice seed.

Keywords: *Tamarindus indica* L., allelochemicals, caffeic acid derivative, agrochemicals, antimicrobial activity

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INTRODUCTION

A variety of agricultural food crops and fruit trees are cultivated throughout the world. Among the several yield limiting factors, soil and air borne diseases and the diseases caused by a variety of bacteria especially in fruit trees are the major constraints in realizing optimum yield potentials of the entire agricultural ecosystem which in turn also cause heavy financial losses annually to the farmers, worldwide. In general synthetic chemicals are advocated for management of these diseases but application of synthetic chemicals provides short term measures as they are reported to induce new strains of pathogens and at the same time also known for their severe environmental hazards. In these respect biological control techniques appears pollution free and economic option to overcome the pathogen problem in agricultural ecosystem as natural agrochemicals are effective and often quickly biodegradable thereby left no toxic residue (Khanh *et al*. 2005). Moreover, at the same time, there is an increasing demand for organically produced and pesticide residue-free food in both international and local markets. Presently allelopathic properties of plants for suppression of plant diseases is considered an effective tool for management of crops therefore, can be exploited successfully for both i.e. management of crops via adopting suitable crop rotation (Anaya 1990) and development of more potent natural agrochemicals based on the identified chemical moieties (Inderjit and Mukerji, 2006). Since, juices from numerous plants or their parts and extracts with organic solvents or root exudates and other secondary metabolites from plants were found to contain various biocidal compounds therefore; some of them are also being utilized as natural herbicides, fungicides, bactericides etc. (Waller, 1987; Putnam and Tang, 1986; Einhellig, 1995; Halbrendt, 1996). In this context several workers have evaluated recently the allelopathic potential of different parts of tamarind tree also. The tree is very well known to release various inhibitors via their roots continuously since from seedling stage to the maturity and after degradation of mature tree leaves. Allelopathic effect of different extract of leaves were studied and very well documented by Mandal and Tapaswi (1997). Later Parvez *et al.* (2003 and 2004) studied the

allelopathic activity of tamarind leaves, bark and seed. All these findings mainly remained focus either on the crude extracts of different plant parts or as organic amendments in field experiments but no study has tried to find out the chemical structure of active compounds. Therefore, an attempt was made in the present study to isolate and characterize the allelopathic compound especially from the ethyl acetate fraction of leaves, which only showed promising activity, up to maximum possible purity and assessing their bioefficacy against a variety of fungal and bacterial organisms and vis-a-vis its impact on rice germination and seedling growth with the main goal to utilize the generated information for the development of natural biopesticide.

MATERIALS AND METHODS

Isolation and characterization of active allelochemicals

To isolate the compounds, green leaves of tamarind were collected and dried under shade. Dried leaves were powdered to very fine dust. Approximately 200 gm of tamarind leaf dust was soaked in 500 ml of methanol for 7 to 10 days. The entire mixture was then stirred at high speed (2500 rpm) for an hour by Mechanical Stirrer (Model Number. DC Stirrer NZ-1000s AC220V, EYELA), and filtered via sintered disc funnel. The brown colored extract obtained was collected and evaporated to dryness under vacuum at low temperature i.e. 45 $\pm$ 2°C. Deep brown colored residue deposited on the surface of evaporator flask was removed by dissolving it in small amount of ethyl acetate and again filtered to trap the non soluble compounds. Clear ethyl acetate solution leached down through the filter was taken to extract the compounds and processed as per the fractionation scheme represented in Figure 1. Prior to fractionation vital information such as total number of compounds present in dried residue and their polarity and concentration etc. were being generated on TLC. Required purity in compound of interest was achieved by repeatedly following the steps of fractionation scheme.

Thin layer chromatography

TLC plates (20 x 20 cm) were used for this study Silica gel G of TLC grade was used as a coating material and plates were coated uniformly with 0.5 mm thick layer of silica gel. A solvent mixture in the ratio of 95: 5:: Hexane : Ethyl Acetate was taken as mobile phase (Stahl, 1969). Plates were loaded with 20 µl solution (500 ppm of TrEAF) and developed up to a height of 18 cm in glass chamber pre-saturated with mobile phase. After complete elution plates were taken out and dried under a stream of hot air. Finally compounds were detected by exposing the plates under iodine vapor or under UV light (365nm).

UV Spectrophotometer Analysis

Helios Gamma UV Spectrophotometer (Model No. NC: 9423 UVG 1002E) was used for recording the λ max of the extracted and purified compound of TrEAF.

MS Analysis

For determination of molecular weight purified TrEAF was analyzed with Mass Spectrometer. Micromass Q-TOF Micro™ Spectrophotometer in its positive ion mode was successfully utilized to analyze the sample.

NMR Analysis

For both ^{1}HNMR and ^{13}CNMR kinds of analysis, 500 MHz NMR SPECTROMETER (MODEL: DRX 500, MFGD. BRUKER) was used. ^{1}HNMR, spectra of TrEAF were recorded on δ ppm (0-10) scale with end of sweep at 0 ppm by using pulse programme Zg and number of scan 64. For ^{13}C analysis Zgdc pulse programme with number of scan IK (1024) was utilized. ^{13}CNMR spectra of TrEAF were also recorded on ppm (0-200) scale with end sweep at 0 ppm. In both the cases samples were analyzed at ambient temperature and $CDCl_3$ was used as solvent for dissolving the compound.

IR Analysis

To confirm the important functional group the extracted and purified TrEAF was analyzed via Infra Red Spectrometer. JASCO-SP-Model No. 410 spectrophotometer was used for analysis of compound. Solid state spectrum was obtained by mixing the required quantity of sample in KBr plate.

Effect of TrEAF compound of Tamarindus indica at different concentrations on the fungi

Inhibition zone test technique was performed for testing the impact of extracted TrEAF against three different fungal species viz., *Aspergillus tamari*, *Aspergillus fumigatus*, *Aspergillius niger*. Few fungal spores of test fungi were transferred in 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. One ml of fungal spore mixed water obtained so was then poured to sterile petridish containing molten PDA and allowed to solidify the plates. Four cups were cut at equidistant position and in these cups 0.5 ml solution of different concentration viz., 500 ppm, 1000 ppm, 1500 ppm and 2000 ppm was added. Treated plates were incubated in BOD incubator at $28 \pm 1^{o}C$ for 24-48 hrs. After 48hr plates were taken out and observations were recorded for colony growth inhibition.

Effect of TrEAF compound of Tamarindus indica at different concentrations on the bacteria

Inhibition zone test technique was done for testing the impact of extracted TrEAF against five different bacterial strains i.e. *Cercina lutia*, *Staphylococcus aureus*, *Micrococcus roseus*, *Streptococcus* sp, *Pseudomonas* sp. Initially the strains of test bacteria were transferred in NA (Nutrient Agar Media) slants and incubated for 24 hrs. Next day, one loop full solution from the slant was added to the nutrient broth and mixed well. The prepared nutrient broth was incubated at $37^{o}C$ for two and half hr. One ml broth was then added to sterile petridish containing molten NA medium and allowed the plates to solidify. After complete

solidification four cups were cut at equidistant position and in these cups 0.5 ml solution of different concentration viz., 500 ppm, 1000 ppm, 1500 ppm and 2000 ppm TrEAF compound was added. Treated plates were incubated at 37$\pm$1°C for 24 hr. after this period observations were recorded on inhibition zone.

Bioassay with TrEAF compound on the germination and subsequent growth of rice

The allelopathic potentials of isolated and purified TrEAF on the seedling growth of rice as well as the effect on germination of rice was determined by laboratory bioassay experiments. Experiments were laid out in replicated Petri plates (90 mm dia) containing a layer of filter papers. 30 mg of TrEAF 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. Nine sets of experiments were performed including control. In the control set 15ml of distilled water was added instead of treated solution. Seeds were surface sterilized with 0.1% mercuric chloride solution, washed with distilled water and placed on a filter paper in Petri dish. After 4 days, shoot length and root length in the control and treated sets were measured (Mandal, 2001).

RESULTS AND DISCUSSION

Isolation and characterization of active allelochemicals

By repeatedly following the proposed scheme (Fig.1) of fractionation we could able to extract the compound of interest i.e. TrEAF in purity of >95% as a single pure compound. Two comparatively more non-polar (R_F Zones 0.99 & 0.87) and two more polar (R_F Zones 0.12 & 0.00) compounds detected on TLC with the major compound of fraction (R_F Zone 0.34) in crude ethyl acetate residue were successively removed by repeatedly following the steps of proposed scheme conducted by dissolving each time the obtained ethyl acetate solid residue (R_F Zone

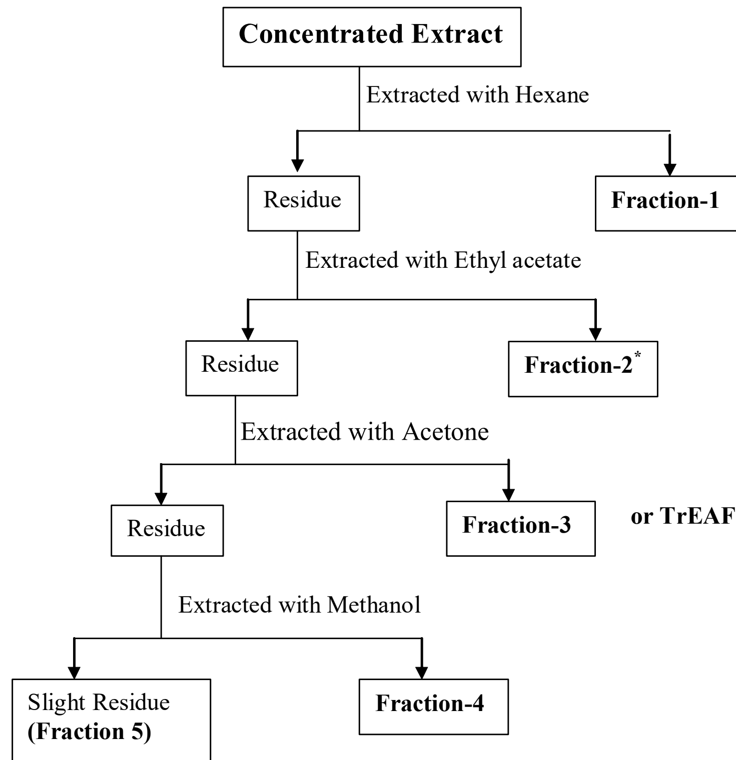


Figure 1. Flow diagram of extraction of compounds from the concentrated lead dust extractof *Tamarindus indica* L.

0.34) in sequentially decreasing amount of ethyl acetate, filtered and dropping the concentrated filtrate drop by drop in approximately 100 ml of hexane or n- hexane. In this process main compound i.e. R_F Zone 0.34 crystallized under hexane and settled in form of white crystals at the bottom of beaker whereas the two more polar compounds i.e. R_F Zones 0.00 and 0.12 retained on the filter paper owing to be their poor or nil solubility in less amount ethyl acetate. The two comparatively more non-polar compounds (R_F Zones 0.99 & 0.87) remained in hexane or n-hexane phase due to their good solubility in hexane and removed by filtration from the compound of interest (R_F Zone 0.34). At least eight time repetitions of the procedure found enough to achieve the required purity in compound of interest.

TLC analysis

TLC methods and other conditions viz., suitable coating material, mobile phase, detecting reagents etc those described under the material and methods section were found suitable for properly eluting the extracted TrEAF. TLC also proves very supportive in removing all other compounds present in TrEAF with the major compound of interest. TLC made a decisive role in selecting suitable solvents for different steps of fractionation scheme because it provided valuable information such as the total number of compounds present in extracted fraction, their concentrations and polarity. Crude ethyl acetate fraction resolved on TLC under five different spots at different RF Zones viz., 0.99, 0.87, 0.34, 0.12 & 0.00. Out of total five only one i.e. 0.34 detected as a major component of the fraction concluded as per the area under spot. On TLC pure compound under iodine vapors showed bright yellow spot whereas under UV light at 365 nm it showed bright fluorescent spot.

MS analysis

Mass spectrum (Fig.2) of purified yellowish TrEAF reveals the molecular weight of 390. The conclusion is drawn based on the appearance of the M+H and M+Na peaks at the spectrum. Though the molecular ion i.e. M^+ peak is not visible in the spectrum but low abundance peak at 391

corresponding to M+H molecular ion and 100% abundance molecular ion peak at 413 (M+Na), exactly at the difference of 23 (molecular weight of sodium) prove vary much decisive in concluding the molecular weight of the said unknown TrEAF compound.

^{1}HNMR analysis

^{1}HNMR spectra of purified compound of TrEAF (Fig.3a and 3b) is constituted of total seven peaks. A set of two duplex peaks at much downfield i.e. δ 7.87 (1H, d, J = 2, 8Hz) and 7.176 (1H, d, J = 10, 8Hz) clear-cut indicates the presence of aromatic ring. Since the aromatic peaks resolved at different chemical shift positions of the spectrum therefore, indicates towards the unequivalent chemical environment of aromatic protons. Which conclude the compound to be of 3, 4 - disubstituted phenyl group derivative. One singlet at δ 3.56 shift position is expected either by the protons of ethereal (CH_2O) group or the unsaturated hydrocarbon viz., HC=C- shifted at 3, 4 place of benzene ring. One triplet at δ 2.27 expected either the presence of acetylinic (CHCH) protons or the protons of methylene or methyl groups of especially those aromatic alkyl side chains retained either ester (-COOAR) or ether (-COAr) as a functional group. Presence of ether already neglected and chance of presence of acetylinic protons is also neglected because no acetylinic carbon peak is detected in the spectrum of ^{13}CNMR. Rests of the peaks i.e. 1.27, 0.9 at much up field of the spectrum are related to the alkyl protons possess different electronic environment. Hence H^{1}NMR spectrum of analyzed unknown compound of TrEAF also support the conclusion of MS analysis and conclude the compound to be of aromatic derivatives in that the aromatic moiety of molecule is heavily shifted by alkane and alkene carbons side chains out of those one also contained esteric group as a functional group.

^{13}CNMR analysis

Figure 4 showed ^{13}CNMR spectra of purified TrEAF compound of

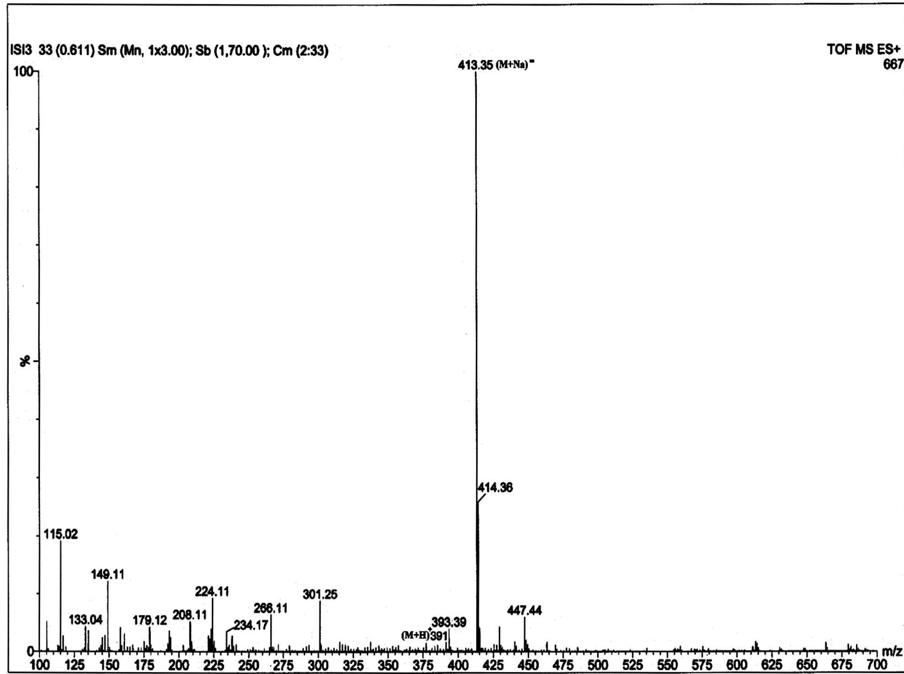


Figure 2. Mass spectra of purified TrEAF compound of *Tamarindus indica* L.

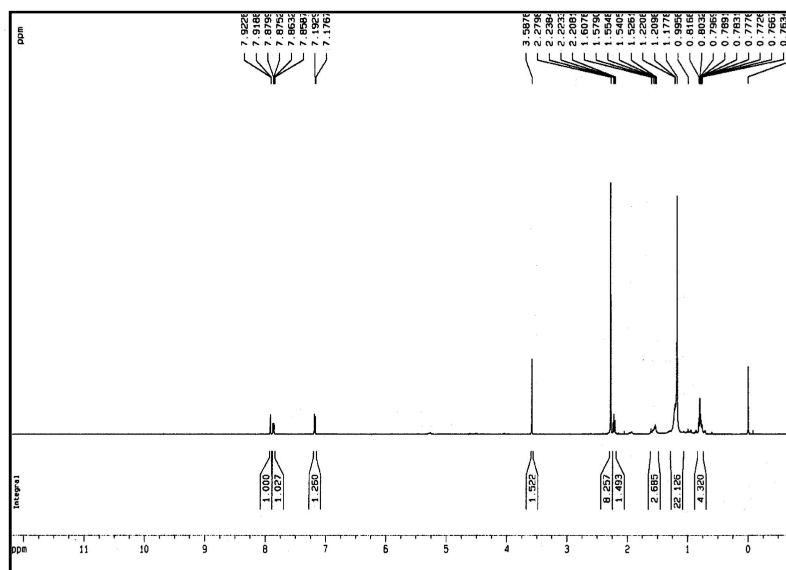


Figure 3a. ^{1}HNMR spectra of purified TrEAF compound of *Tamarindus indica* L.

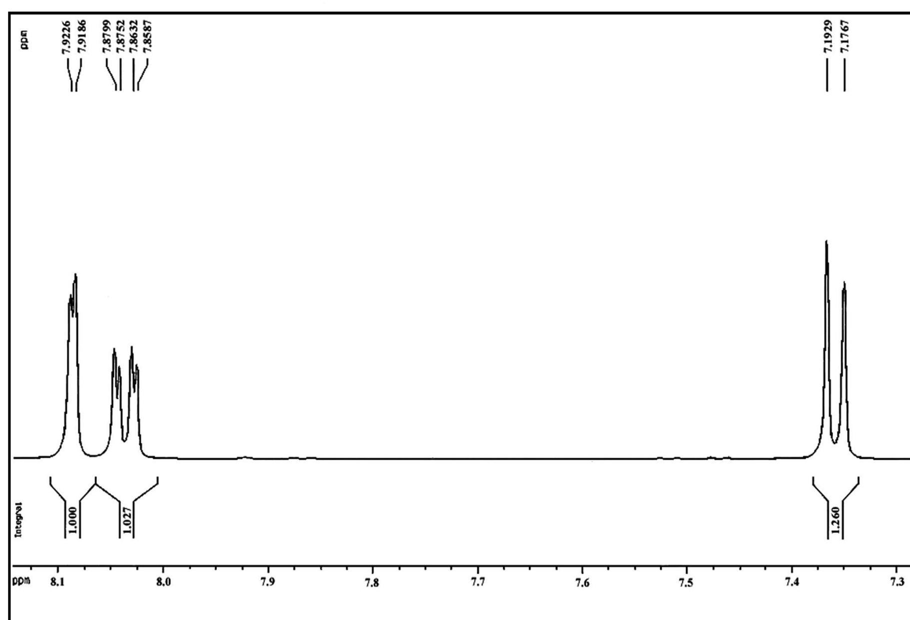


Figure 3b. Expanded ^{1}HNMR spectra of purified TrEAF compound of *Tamarindus indica* L.

Tamarindus indica. One small peak at δ 174ppm definitely pertains to the carbonyl carbon of ester group. whereas, relatively large two peaks at δ146 and δ 145 pertains the presence of dihydroxy benzyl ring in the compound. Two more peaks at δ 138 and 130ppm are due to the other carbons of the aromatic ring. Two peaks at δ 124 and 121 are due to the alkenes carbon. Peaks at δ 77 are coming by the $CHCl_3$ impurities present in traces in $CDCl_3$ (solvent used for dissolving the compound). Rests of the peaks at up field (δ1- 50) of spectrum are due to the terminal methyl and different methylene (CH_2) carbons of substituted side chains, facing different kind of chemical environment into the molecules. Therefore ^{13}CNMR analysis also proves it's to be a highly substituted aryl ester.

IR analysis

Figure 5 showed the IR spectra of purified TrEAF compound of *Tamarindus indica*. All aromatic compounds generally show a most characteristic set of four bands pertains to the C=C stretching near 1600 cm^{-1}, 1580 cm^{-1}, 1500 cm^{-1} and 1450 cm^{-1} hence a set of three peaks at 1588 cm^{-1}, 1522 cm^{-1} and 1450 cm^{-1} in the observed spectrum of extracted compounds are due to the C=C stretching of benzene ring therefore confirms the aromaticity into the extracted molecule. Appearance of a strong and very sharp band at 1736 cm^{-1} pertains to C=O stretching in carbonyl compounds (1650-1950) (Silverstein *et al*, 1963). Since esteric carbonyl carbon shows the characteristic ν C=O absorption at 1750-1735 cm^{-1} hence appearance of sharp band at 1736 cm^{-1} certainly confirms the presence of ester group. Moreover two bands at 1269 cm^{-1} and 1168 cm^{-1} are due to the stretching vibrations in C-O-C bond, hence again left no doubt to conclude the presence of ester group in the compound. Symmetric and asymmetric C-H stretching vibrations frequencies of aromatic, alkenes and alkanes got mixed in the present spectrum and appeared as broad bands between the region 2924 cm^{-1} to 2856 cm^{-1}. The spectrum at 1348, 1269, 1168 cm^{-1} indicates the absorption of long chain esters hence confirm the presence of alkyl chain retained esteric group.

Though the IR, MS, ^{1}HNMR and ^{13}CNMR analysis revealed all important clues about the chemical structure of extracted TrEAF but to elucidate the exact chemical structure there is a need to generate these entire spectrums by analyzing absolute pure compound. Moreover there

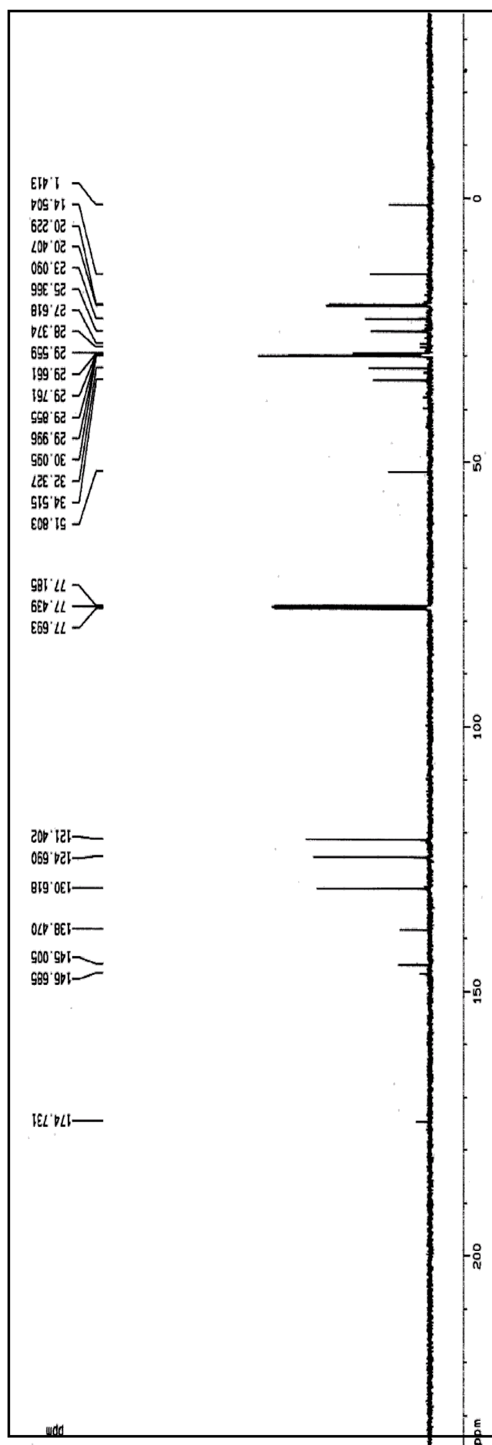


Figure 4. ^{13}CNMR spectra of purified TrEAF compound of *Tamarindus indica* L.

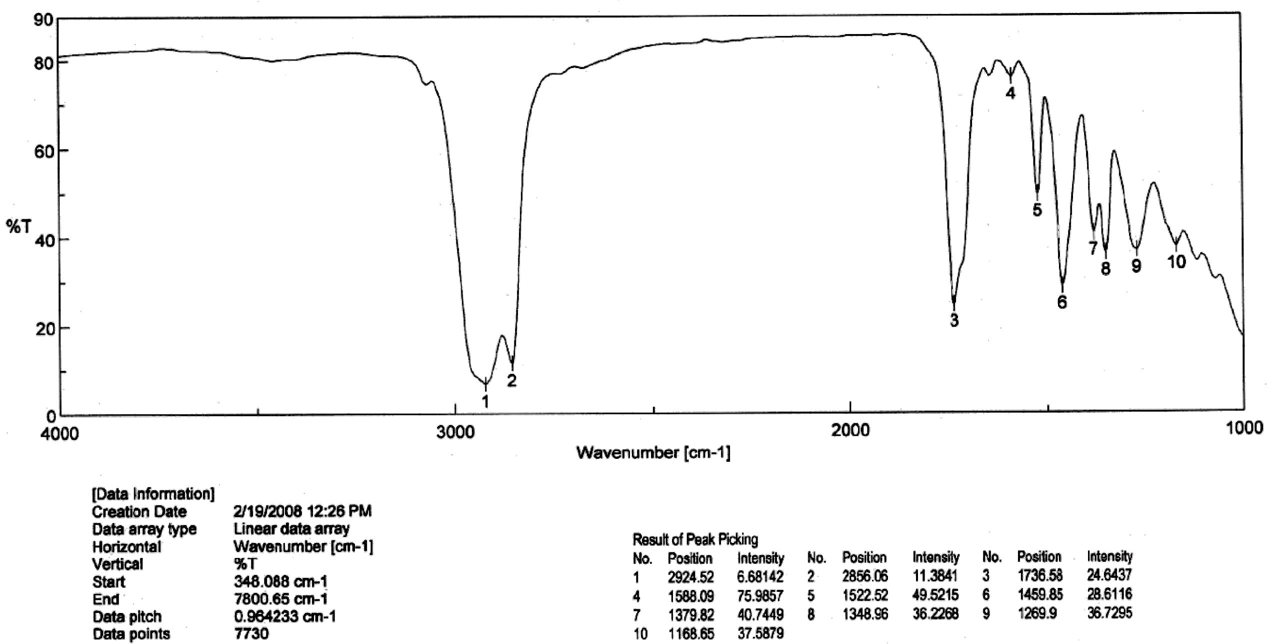
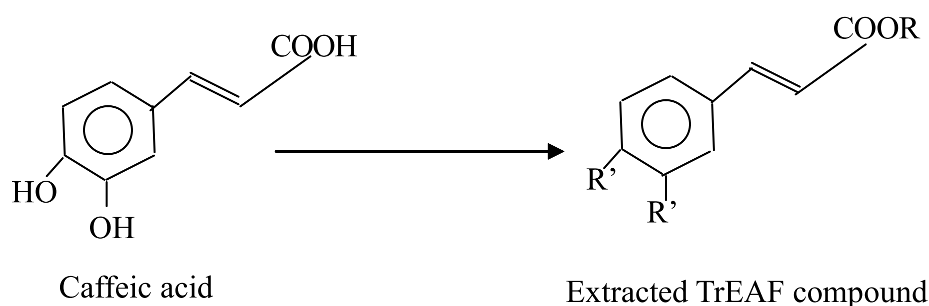


Figure 5. IR spectra of purified TrEAF compound of *Tamarindus indica* L.

is also need to generate ^{1}HNMR spectrum of absolute pure compound in its different modes viz., by increasing field strength, double resonance and by lanthanide shift reagents. Off resonance proton decoupling ^{13}CNMR spectrum of pure compound is also needed. Though the work on absolute purification of all the compounds of tamarind is presently going on in our laboratory even though the present results clearly indicates that the extracted compound could be a secondary metabolic produce of caffeic acid via some enzymatic biochemical reaction. It can easily be understood that the hydroxyl (OH) group present at the meta and para position of benzene ring wholly replaced by some alkyl chains as IR spectrum of compound showed no hydrogen bonded stretching in the region of 3400-3200 cm^{-1}. Moreover no broad peak of OH protons between δ 4.5-6 chemical shift regions of ^{1}HNMR spectrum is detected. Therefore, these two observations clearly indicate the replacement of hydroxyl protons. Alkyl chain also shifted by replacing the hydrogen atom of carboxylic group and made it's an ester because in this case also a very broad peak expected by the hydrogen bonded stretching (3000-2500 cm^{-1}) vibrations of carboxylic acid group under IR and out of scale peak at about δ 12-13 of ^{1}HNMR spectrum were found absent. Chemical structures of caffeic acid and tentative structure of extracted TrEAF compound are giving below.



Caffeic acid

Extracted TrEAF compound

Effect of TrEAF compound of Tamarindus indica at different concentrations on the fungi

In the inhibition zone test, three fungal species viz. *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillius tamarii* showed different diameters of inhibition zone at different concentrations of TrEAF compound. *Aspergillius tamarii* is highly sensitive to TrEAF compound than *Aspergillus niger* and *Aspergillus fumigatus*. *Aspergillius tamarii* is much more sensitive to TrEAF compound at 1500ppm concentration (Fig.6a and 6b).

Effect of TrEAF compound of Tamarindus indica at different concentrations on the bacteria

In the inhibition zone test, *Cercina lutia*, *Staphylococcus aureus*, *Micrococcus roseus*, *Streptococcus* sp and *Pseudomonas* sp revealed different diameters of inhibition zone at different concentrations of purified TrEAF compound. *Cercina lutia*, *Staphylococcus aureus*, *Micrococcus roseus* and *Pseudomonas* sp were much more sensitive to TrEAF compound than *Escherichia coli*. No effect of TrEAF was detected in *E.coli* at concentration of 1000ppm (Fig.7a and 7b).

Effects of TrEAF compound of Tamarindus indica at different concentrations on the germination and subsequent growth of rice

This TrEAF compound showed inhibitory activity in all concentrations on rice seeds (Fig.8). At concentration of 1000ppm, it showed 53.82% inhibition on shoot length and 58.85% inhibition on root length. At 500ppm concentration, 29.017% inhibition in shoot length and 38.82% inhibition in root length has been detected. It revealed 27.49% inhibition in shoot length and 31.06% inhibition in root length at 250ppm concentration. At a concentration of 125ppm, 21.07% inhibition in shoot length and 22.68% inhibition in root length were noticed. Very slight stimulation (7.06%) in shoot length was observed at 7.81ppm concentration. This compound did not show any effect on the germination of rice seed.

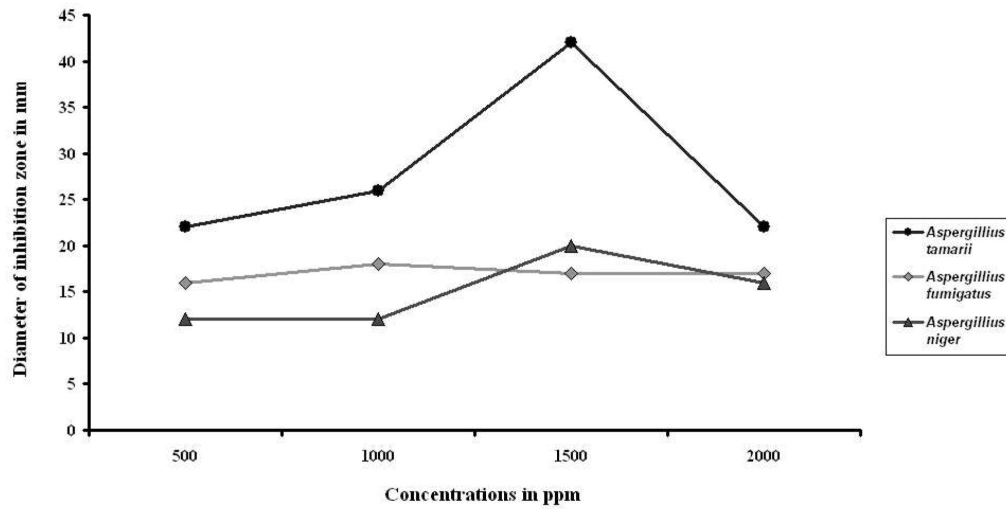


Fig. 6a. Antifungal activity of TrEAF compound of *Tamarindus indica* at different concentrations on *Aspergillus tamarii*, *Aspergillus fumigatus, aspergillus niger.*

Figure 6b. Effects of TrEAF compound of *Tamarindus indica* L. at different concentrations on the fungus, *Aspergillus tamarii* and *Aspergillus niger.*

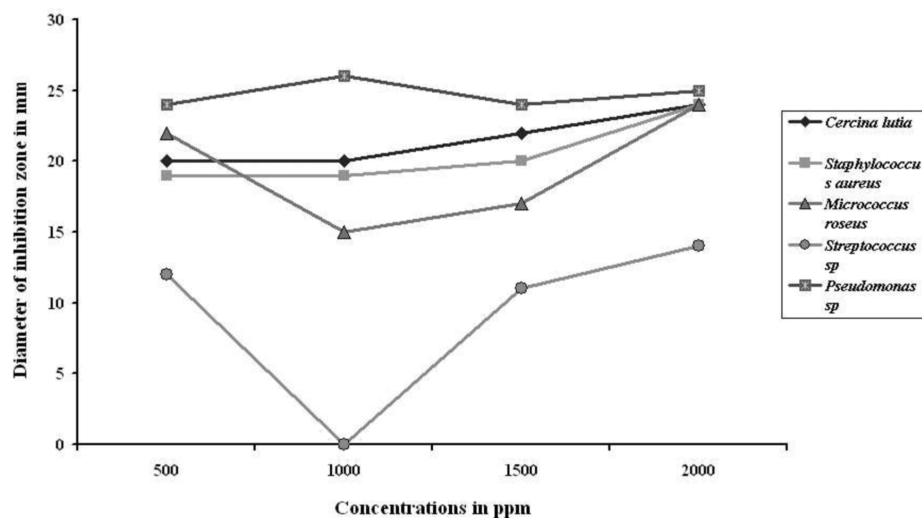


Figure 7a. Antibacterial effects of TrEAF compound of *Tamarindus indica* L. at different concentrations on *Cercina lutia, Staphylococcus aureas, Micrococcus roseus, Streptococcus* sp. and *Pseudomonas* sp.

Figure 7b. Effects of TrEAF allelochemicals at different concentrations on the bacteria, *Cercina lutia, Staphylococcus aureas, Micrococcus roseus, Streptococcus* sp. and *Pseudomonas* sp.

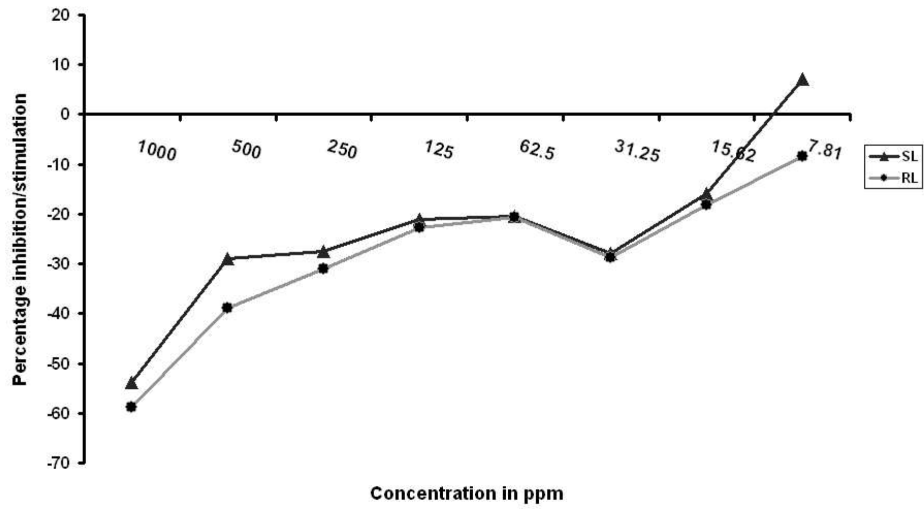


Figure 8. Effects of TrEAF compound of *Tamrindus indica* L. at different concentrations on the germination and subsequent growth of rice

CONCLUSIONS

Tamarindus plants shed leaves in all season and vegetation under the tree is very sparse due to the inhibitory effects of leaf leachates. A potent biopesticide with strong antifungal and antibacterial activity has been isolated and purified from the Ethyl Acetate Fraction (TrEAF) of Tamarind leaves. Chromatographic analyses of TrEAF compound revealed that the compound is putative caffeic acid derivative. There are no reports on the presence of caffeic acid in Tamarind leaves. Caffeic acid is a derivative of cinnamic acid, which is derived from the shikimic acid pathway and is widespread in plants (Harborne, 1984).

Allelochemicals for the synthesis of the biopesticides and growth promoters is emerging as an important research area. Since most of the allelochemicals are known to be toxic to a variety of weeds and pests, they can be exploited as natural pesticides (Miles *et al*, 1993). The development of natural products such as herbicides, fungicides, and their role in biological control of plant diseases, indicates a reduction in environmental and health hazards. This active ingredient of TrEAF compound revealed defense activity to various fungal and bacterial species but no effect on the germination of rice seed. This compound may be utilized as bioactive fungicide and bacteriocide in agriculture. The present invention provides natural biopesticides which can replace synthetic chemicals and are much safer, from a health and environmental point-of-view.

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