Trakia Journal of Sciences, Vol. 8, No 2, pp 1-15, 2010

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

Original Contribution

PRO-ANTIOXIDANT ACTIVITIES OF FRACTIONS OF A NOVEL CAMPTOTHECIN-PRODUCING ENDOPHYTE (*ENTROPHOSPHORA INFREQUENS*)

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ABSTRACT

PURPOSE The overexploitation of wild medicinal flora for use in modern medicinal systems has led to a significant threat to the endemic medicinal flora. It is, therefore, imperative to develop biotechnological interventions to ensure regular availability of these bioactive compounds. METHOD: In the present study, with a view to developing a biotechnological alternative, an endophyte- *Entrophosphora infrequens* was isolated from the inner bark of the medicinal plant *Nothapodytes foetida*. The endophyte was authenticated using CLUSTAL W-based phylogenetic analysis; subsequently its independent culture and unequivocal demonstration of production of camptothecin (CPT), an anti-cancer secondary metabolite was proved. RESULTS: A comparison was made between CPT- (CPT devoid) and CPT+ (CPT enriched) fractions, followed by evaluation of their pro-antioxidant (biphasic) activity in lipid phase. Mechanistic analysis revealed a significant ($p< 0.05$) prooxidant activity in the CPT+ fraction (50µg/ml) and could be attributed to the presence of CPT, as compared to CPT- fraction that exhibited antioxidant behavior at higher concentrations tested (100–1000µg/ml). Presence of CPT also resulted in significant ($p<0.05$) enhancement in net electron donation potential ($R^2 = 0.912$) and free radical scavenging potential ($R^2 = 0.963$). Both the fractions exhibited comparable nitric oxide modulatory activity (~> 65%), while Ferric Reducing Ability in Plasma (FRAP) equivalence value of CPT+ fraction was found to be 1.02 ± 0.04 times higher as compared to CPT- fraction (time points x, 8x and 16x; x= 300s), explaining its prooxidant behavior at lower concentrations. CONCLUSIONS: In conclusion, the prooxidant activity positively correlated with the presence of CPT, acting in synergism/antagonism with other active constituents. The contrasting potential of both fractions indicates its usage as an anti-cancer drug and as antioxidants and/or radiomodulator.

Keywords: Prooxidant, Antioxidant, Radiomodification, Endophytic, Anti-cancer, *Nothapodytes foetida, Entrophosphora infrequens*

INTRODUCTION

Microorganisms have been evaluated and screened for the presence of a plethora of bioactive compounds. Such studies have led to practical development of a number of drugs, many of which find use in modern systems of medicine. Endophytes are microorganisms that colonize the tissues of living plants without posing any harm to the host. Plants have developed different types of mutual relationships with other species, including endophytes. The endophytes have been explored as potential sources of novel natural

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products for medical, agricultural, and/or industrial exploitation in recent years (1, 2). This offers significant scope to explore the potential of such species as a prospective source of bioactive compounds. Some plants synthesizing biologically active natural products have endophytes associated in their biological niche and such endophytes have been shown earlier by several workers to produce similar natural secondary metabolites e.g., endophytic fungi *Taxomyces andreanae,* isolated from *Taxus brevifolia; Seimatoanthlerum tepuiense* from the Venezuelan-Guayana system, *Periconia sp.* from *Torreya grandifolia, Tubercularia sp.* strain TFS from *Taxus mairei* have been shown to produce Taxol (3-6). Endophyte-based production of podophyllotoxin has been reported earlier by our group (7). Secondary metabolite synthesis in the plant is a dynamic process involving a cascade of reactions, yet the amount in which they are produced in the natural plant populations is usually abysmally low which necessitates development of alternative approaches, including the use of biotechnological interventions to augment their production. It is possible to produce secondary metabolites in plant cell cultures or co-culture with fungal cultures; however a major disadvantage is limited production in such cultures and upscaling is usually difficult and not an economically feasible option (8, 9).

Several reports indicate that population of endophytic microorganisms produce bioactive molecules that exhibit antineoplastic, antibacterial, antiviral, antioxidant, antidiabetic properties, similar to ones produced in the host plant (2, 7, 10-14).

The antineoplastic activity of camptothecin (CPT) **(Fig. 1a)**, a pentacyclic quinoline alkaloid, is well known. CPT inhibits the intracellular enzyme topoisomerase I, which is involved in relieving tension on DNA strand generated during replication (15). The entrapment of CPT with enzyme leads to the formation of a complex that stalls the replication fork producing DNA double strand breaks (DSBs) (16). CPT and its synthetic derivatives exhibit potent anti-neoplastic activity and are indicated in treating skin ailments (17). Several semi-synthetic derivatives of CPT have been generated, out of which topotecan and irnotecan have been shown to be effective against colon and ovarian cancers (18). The antiviral activity of CPT against herpes virus/influenza virus is also reported (19).

Apart from *Camptotheca acuminata,* CPT is also found in *Nothapodytes foetida* (Wight) Sleumer, *N. collina*, *N. obscura, N.obtusifolia, N. tomentosa* and *Ophiorrhiza pumila*, to name a few. The plant-based yield of CPT is very low and overharvesting of the medicinal plant for production of such drugs often leads to extensive depletion of their natural population. Attempts have been made to synthesize CPT, but again the process involves several steps and is commercially non-viable (20). Puri and co-workers isolated the endophyte *Entrophospora infrequens* from *Nothapodytes foetida*, which showed that it produces camptothecin (CPT) when grown *in vitro* and showed its immunomodulatory properties (11, 12).

The present study was taken up with fractions of this unique endophyte to investigate their comparative pro-antioxidant activities in different phases (aqueous and lipid phase) and free radical scavenging potential. Redox balance of antioxidants and free radicals maintains the biochemical equilibrium. The disturbance caused by radiation stress requires augmentation of exogenous antioxidants by supplementation, while in case of cancer the preferential killing of cancerous cells is primarily based upon the prooxidant potential of the drug. Such properties refer to the radiomodifying properties. The prime focus of the present study was to evaluate the effect of presence of camptothecin in modification of biological response relevant to radiation protection (~antioxidant) and its anti-cancerous potential (prooxidant).

METHODS

Plant Material Collection, Identification and Authentication

Plant material was collected from the plant growing in the botanical garden of Indian Institute of Integrative Medicine (IIIM), Jammu, India (formerly Regional Research Laboratory). The plant was identified and authenticated on the basis of botanical characteristics by an experienced botanist. The inner bark of *Nothapodytes foetida* was used for the isolation of the endophyte.

Isolation and Establishment of *in vitro* Culture of the Endophyte

The endophyte (fungi) was collected from the inner bark of *Nothapodytes foetida* using a method described earlier (12). The culture was grown in liquid broth under surface culture conditions. Mycelia and broth were separated by filtration. Mycelia were thoroughly washed with sterile double-distilled water and homogenized in a cell disintegrator. Both cell homogenate and cell free broth were extracted four times with equal volume of L.R. grade chloroform:methanol (4:1 v/v) solvent mixture. Solvent was stripped off in a rotary evaporator leaving behind organic residue. The residue on application on silica gel TLC plates (Merk K GaA, 64271 Darmstadt, Germany) was run in (9:1 v/v) chloroform: methanol solvent system exhibited spots which were super imposable with the standard camptothecin [CPT]. The spots were visualized under UV.

Endophyte Authentication using Homology Modelling

Microscopic slides were prepared, stained using lactophenol cotton blue and were examined under light microscope (Olympus, USA). Photographs were taken by using a digital camera (Camedia camera, C-2100 ultra zoom, Tokyo, Japan). The total genomic fungal DNA was extracted by CTAB method. For DNA extraction, the fungus was grown in 100 ml Sabouraud Dextrose Agar at 28°C with constant shaking for 3 days.100 mg mycelial biomass was taken following washing (two times) with sterile Tris-EDTA buffer, 6ml of CTAB extraction buffer and 60µl of β-mercaptoethanol were added. After mixing, the mixture was incubated at 65°C for 45 min and the contents cooled to room temperature. This was followed by extraction with equal volume of chloroform and centrifugation at 10,000 g for 10 min. Equal volume of isopropanol was added to the supernatant and mixed gently and DNA spooled out by glass rod. The DNA pellet was washed with ice-cold 70% ethanol (v/v). The DNA pellet was vacuum dried and dissolved in 100µl of TE (pH 8.0). The endophytic fungus was identified by analysis of the large subunit of ribosomal genes. The large subunit of ribosomal gene was amplified and sequenced using MICROSEQ D2, large subunit (LSU~300bp) fungal rDNA sequencing kit (Applied Biosystems, USA). The amplified products were purified utilizing Microcon columns (Millipore, USA), and sequenced using ABI Prism310 genetic analyzer (ABI, USA) as per the manufacturer's instructions. The DNA sequences ~300 and 500 bases, thus obtained were submitted to Genbank for homology studies by BLASTN program followed by CLUSTAL W based analysis (21). The ribosomal gene database (http://rdp.cme.msu.edu and http//ncbi.nim.nih.gov) were accessed and sequence alignment was used as an underlying basis to identify the fungus.

Maintenance of Growth of the Endophytic Organism

The endophytic fungus was grown in Sabouraud broth consisting of dextrose (4%) as sole carbon source, and peptone (1%) as nitrogen source. The endophyte was grown in 1000ml Erlenmeyer flasks each containing 200 ml liquid broth (pH 5.6) for a period of seven days at 28±2°C, on an incubatory shaker (New Brunswick, USA; 220 rpm). Spore suspension was used as inoculums. 10^6 spores / ml were inoculated in each flask; the samples were harvested at different time points and subsequently extracted. Solvent was stripped off using rotary evaporator leaving behind organic residue. The first sample was collected immediately after inoculation (zero hour) and subsequent samples were collected every 24 h up to 7 days. The samples filtered to separate mycelia and broth, and the fresh biomass was determined. After repeated washing with sterile demineralized water, the residual medium was removed. The moisture content, final pH and dry weight was also determined for each sample. The experiments were performed in triplicate and were repeated six times.

Mycelia Extraction and Preparation of Fractions

The mycelia and broth were separated by filtration. The mycelia pellet was homogenised and cell homogenate extracted four times with a mixture of chloroform:methanol (4:1 v/v). The chloroform: methanol extract of mycelia was subjected to column chromatography using silica gel and the elution was done using hexane, ethyl acetate, chloroform and methanol in appropriate proportion and sequence. The two fractions were subsequently selected for evaluation of biological activity. The first one was chloroform:methanol (4:1, v/v) extract of fungal mycelia (incubation period –4 days), while the second fraction of the parent extract was obtained by elution of parent extract in methanol. The fractions thus obtained were used for detection of the camptothecin using

HPLC. The former fraction was coded as CPT+ while the other was designated as CPT-.

HPLC based Analysis of Fungal Camptothecin

HPLC separation was performed using Luna RP18 column (2mm i.d., length 150 mm, particle size 3μm) and a safety guard (Phenomenex, Torrance, CA, USA) at 30°C. The mobile phase of water (A) and acetonitrile (B) was used in the following manner (0-5 min 90 % A and 10 % B, 5-20 min 40 % A and 60 % B, 20-30 min 2% A and 98% B, 30-32 min 2% A and-98 % B and 35-37 min 90 % A and 10% B v/v) at a flow rate of 200μl min^{-1} detection: UV at λ = 256 nm, $t_{R.}$ =20.15 min. 10 μl of sample was injected in $CHCl_3$ / MeOH (4:1).

Ammonium Thiocyanate Assay - Based Study of Pro-antioxidant Activity in Lipid Phase

The biphasic activity of CPT- and CPT+ was evaluated following the ammonium thiocyanate assay in which the linoleic acid pre-emulsion (lipid phase) was prepared as described earlier (22). A pre-emulsion of linoleic acid was prepared by mixing 3 volumes of linoleic acid in 200 volumes of 30 % (v/v) ethanol. An equal volume of Tween-20 (~ linoleic acid) was also added as an emulsifying agent. The emulsion of linoleic acid was subjected to slow auto-oxidation as well as exposed to radiation and thus, peroxyl ions levels build up with time. A time-dependant study was performed and samples were exposed to supra-lethal radiation i.e 0.25 kGy using ^{60}Co gamma chamber (Gamma cell 5000, Bhabha Radiation Isotope Technology, Mumbai) at a dose rate of 2.93 kGy/h after 24 h of incubation. The monitoring was performed over the period of few days till the control value (without any extract) dropped. Aliquots were taken at different time intervals up to 52hr. In the monitoring assay, the peroxyl ions were made to react with ammonium thiocyanate (30%) and ferrous chloride (0.1%) in a total volume of 200μl to form red colored ferrothiocyanate readable at 500nm against ethanol in the reference cell. The increase/decrease in peroxyl levels (which is in direct proportion with absorbance) with respect to control exhibits the prooxidant and antioxidant activity in lipid phase respectively.

Electron Donation Potential Directly Related to Increase in Absorbance

The electron donation potential of CPT- and CPT+ was evaluated using ferricyanide reduction assay as described earlier (22). The reaction sample (50μl) of the two extracts was mixed with 200 μl each of 0.2 M phosphate buffer (pH 6.5) and 0.1 % potassium ferricyanide and incubated at 50°C for 20 min. 10% of trichloroacetic acid was added and the mixture was centrifuged at 3000xg. The supernatant was collected and 0.1 % ferric chloride (100μl) added followed by dilution with double distilled demineralized water (500μl). The resulting mixture was re-incubated at 37°C for 10 min, and the absorbance was recorded at 700nm. The solution devoid of test extract was taken as blank. Increased absorbance was indicative of increased electron donation potential.

ROS and RNS Quenching Potential

The super oxide ion quenching ability of CPT- and CPT+ was determined using nitrobluetetrazolium reduction assay (23). Different concentrations of the two extracts were taken and mixed with sodium pyrophosphate buffer (0.052M, pH 8.3) and phenazine methnosulfate (186 μM). Nitrobluetetrazolium (300μM) was added to the above solution and the final volume adjusted to 3 ml. The reaction was initiated by adding NADH (780μM) and the solution was incubated at 37°C for 3 minutes. The reaction was terminated by adding glacial acetic acid and 4 ml of n-butanol. The reaction mixture was allowed to stand for 10 minutes at room temperature, n-butanol layer was separated by centrifugation (1000x g), and the intensity of chromogen (in the n-butanol layer) was measured at 560nm. The percentage inhibition of formation of chromogen exhibits free radical scavenging activity of the extracts.

In a separate experimentation, the varied concentrations of CPT- and CPT+ were mixed with sodium nitroprusside (5mM; generates nitric oxide at physiological pH that interacts with oxygen to generate nitrite ions), and the volume made up to 1ml using phosphate-buffered saline as described earlier (24). The reaction mixture was incubated at 25°C for 150 min followed by addition of Griess reagent. The nitric oxide modulatory activity was evaluated as increase/decrease in percent absorbance of the complex formed by diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethylen-ediamine recordable at 546 nm (25). The nitric

oxide modulatory activity can be calculated as :

$$\% \text{ Activity} = \frac{\text{O.D.}_{\text{control}} - \text{O.D.}_{\text{sample}}}{\text{O.D.}_{\text{control}}} \times 100$$

Ferric Reducing Ability- Kinetic Analysis

The assay was proposed by Benzie and Strain, 1996 and modified by Firuzi and his coworkers (2005) to micro-FRAP assay (26, 27). Initially, the FRAP solution was prepared by mixing 10 ml of Acetate buffer (300mM; pH was adjusted using acetic acid) with 1ml of ferric chloride hex hydrate (20mM) and 1 ml of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ dissolved in 10 ml hydrochloric acid (40mM). 25µl of each product under investigation at different concentrations (1-2000µg/ml) was placed in quadruplicate in a 96-well micro plate reader. To each of these triplicate samples, 175µl of freshly prepared (warmed at 37°C) FRAP solution was added, while to the fourth sample, same volume of acetate buffer was added to serve as blank sample. In the fifth row of micro-plate reader, 25µl of the solvent system was taken and similarly FRAP solution was added to each of the solvent blank. The absorbance at 595nm was monitored by a micro plate reader [Bioteck Instruments Inc. (USA)] at different time intervals up to 150 min. The absorbance of the blanks and that of mixture of 175µl-FRAP solution plus 25µl solvent system were subtracted from the absorbance of the samples at each time interval to calculate the absorbance change (ΔA). All substances were tested at least in triplicate at 37°C as described above. The FRAP Equivalence value at the time interval t (FRAP value (28)) was calculated as FRAP Equivalent Value (28) (M) = Ratio of absorbance change at time (t) of test sample at particular concentration that corresponds to same absorbance change at same time (t) of standard.

Statistical Analysis

All the results are expressed as mean ± standard deviation (s.d.). Pro-antioxidant activity in lipid phase was analyzed using One-Way ANOVA analysis of variance followed by Tukey's HSD test (n= 5; post-hoc test). Analysis of the peroxyl levels was performed at different time intervals. The comparative analysis of means was done using Student's t-test. Statistical significance was designated as $p < 0.05$ (95 % significance).

RESULTS AND DISCUSSION

Establishment of Endophyte and Characterization of Camptothecin in Endophytic Fractions

Natural plant products yield numerous bio-active compounds, many of which find use in medicine for a wide variety of applications. Since most secondary metabolites are high-value, low-volume compounds, they are produced by plants in miniscule amounts and plant biotechnological interventions too are not very effective on commercial scale. To overcome some of the limitations, the alternative choice is to identify microorganisms sharing the same ecological niche as that of medicinal flora for enhanced production of secondary metabolites. In some cases, these microorganisms have adapted and acquired the ability to produce similar, often same, valuable secondary metabolites independently with the passage time. In our earlier study, we identified one such species i.e., *Entrophosphora* species, an endophyte from *Nothapodytes foetida* (12). The ribosomal RNA based confirmation of identity of endophyte is one of the well accepted mode of evaluation of phylogenetic position of collected endophyte and was done to confirm the identity of the endophyte **(Fig. 1b).** The identification of the endophytic fungi collected was established on the basis of LSU rDNA sequence (5'GGAAA …. CTTGA 3') alignment and BLAST analysis using specific data bases **(Fig. 1b)** and thereby confirmed to be *Entrophospora infrequens* consistent with earlier observations in the previous study (12). The CLUSTAL W-based phylogenetic analysis was carried out to analyze the level of divergence and to establish the percentage identity of *Entrophospora infrequens* with respect to level of divergence, as compared to *Rhizopus oryzae* as illustrated in **Fig. 1c**. The identification of endophyte led us to develop its independent culture, followed by the identification of main bioactive constituent. The culture parameters were standardized as described in the previous study and time-dependent growth vs. production was analyzed. The growth kinetics of *Entrophospora infrequens* is given in Table-I. An increase in dry weight of the mycelia was observed till 168 hrs and thereafter it declined. The mycelia was collected at different time intervals of 48, 96, 144, 192 hr respectively and screened for camptothecin content using HPLC and maximum production of

camptothecin was observed at 96 hours in terms of dry weight of the mycelia **(Table-I)**. The search for *N. foetida* associated microbes that produce camptothecin is justified by previous examples of plant associated microbes producing plant compounds, such as gibberellins (29). The production kinetics of camptothecin preceded growth in shake flasks, thus the fraction collected at 96 hrs from the chloroform:methanol (4:1) extract was designated as CPT+ as it contained camptothecin, and was used as positive control for CPT. The identification of camptothecin was established using HPLC based gradient analysis (water : acetonitrile), by comparing the standard peak of camptothecin at 24 min **(Fig. 1d)** with respect to the corresponding prominent peak in CPT+ at the same retention time **(Fig. 1f)**. On the other hand, in order to establish the correlation between biological activities and CPT, a negative fraction (CPT-; CPT devoid fraction) was also prepared separately using methanol as eluent, designated as CPT- as illustrated in **Fig.1e**. The fingerprint analysis of CPT- exhibits two prominient peaks at 5.66 and 31.89 min respectively **(Fig.1e)**. HPLC-based characterization of camptothecin in CPT+ fraction **(Fig.1f)**, as compared to CPT- fraction **(Fig1e)**, indicated the role of solvent system in elution of the particular bioactive compound. It exhibits the industrial potential for production of CPT in enormous amount without endangering the endangered flora. Such observations are in consistency with reported literature on *Nothapodytes foetida* as a rich source of the potent alkaloid camptothecin and 9-methoxy camptothecin (30). Camptothecin is well known for its anti-cancerous potential and its potential to act as a radiomodifier has been established by experimentation. Thus, the comparative bioactivity analysis was carried out using CPT- and CPT+ fractions.

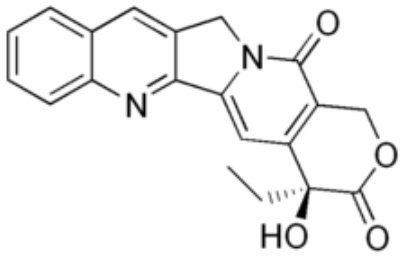

Fig. 1 a

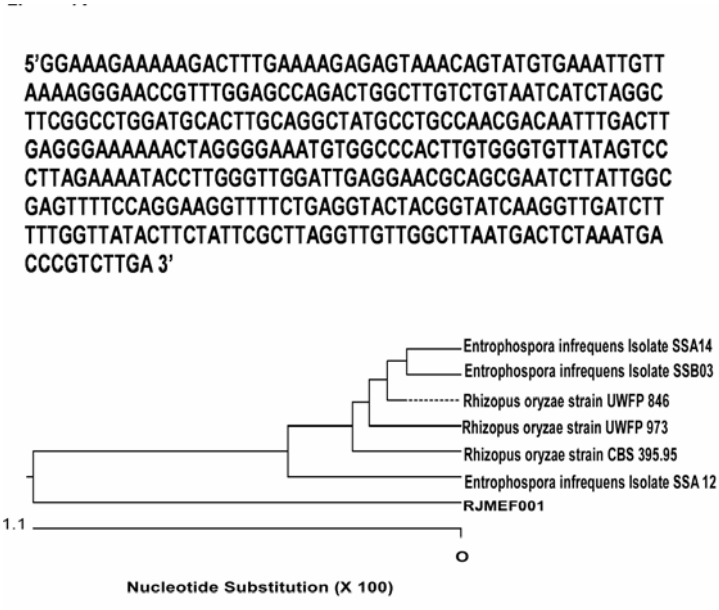

Fig. 1 b.

Percentage Identity

	1	2	3	4	5	6	7	
1		99.2	98.9	99.4	98.9	98.4	98.6	1
2	2.0		99.2	99.9	99.5	98.9	99.2	2
3	2.3	0.1		99.7	99.7	98.7	99.5	3
4	1.7	0.1	0.3		99.2	99.2	98.9	4
5	2.3	0.3	0.0	0.5		98.9	99.7	5
6	2.3	0.8	1.1	0.5	1.1		98.7	6
7	2.6	0.5	0.3	0.8	0.3	1.3		7
	1	2	3	4	5	6	7	

Divergence

1 RJMEF001
2 Rhizopus oryzae strain UWFP 973
3 Rhizopus oryzae strain UWFP 846
4 Rhizopus oryzae strain CBS 395.95
5 Entrophospora infrequens Isolate SSA12
6 Entrophospora infrequens Isolate SSA 14
7 Entrophospora infrequens Isolate SSB03

Fig. 1 c.

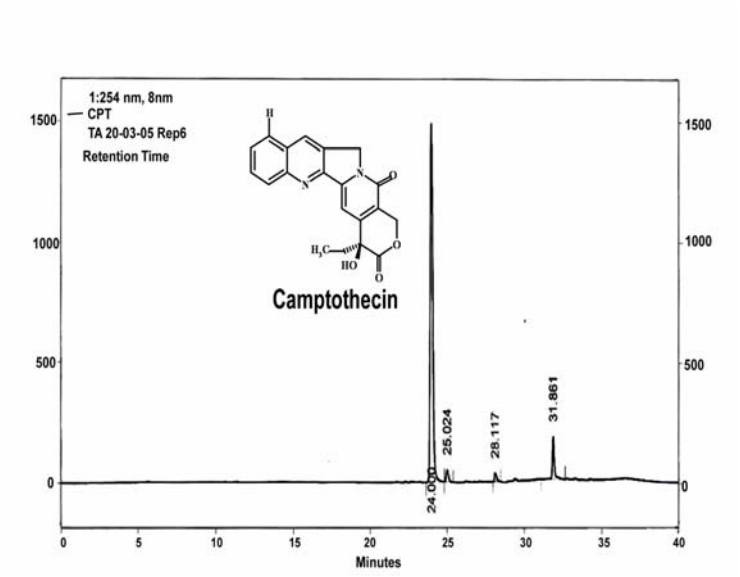

Fig. 1 d.

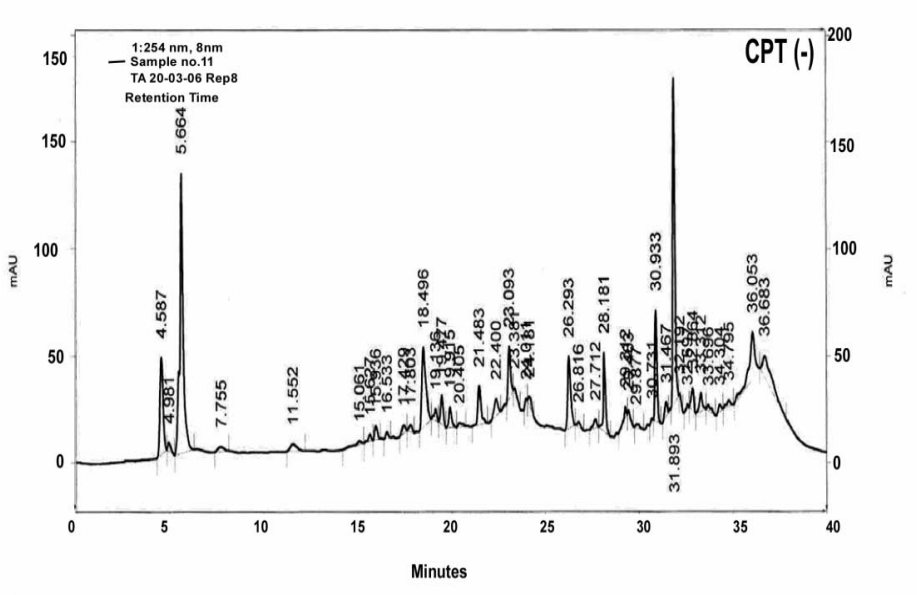

Fig. 1 e.

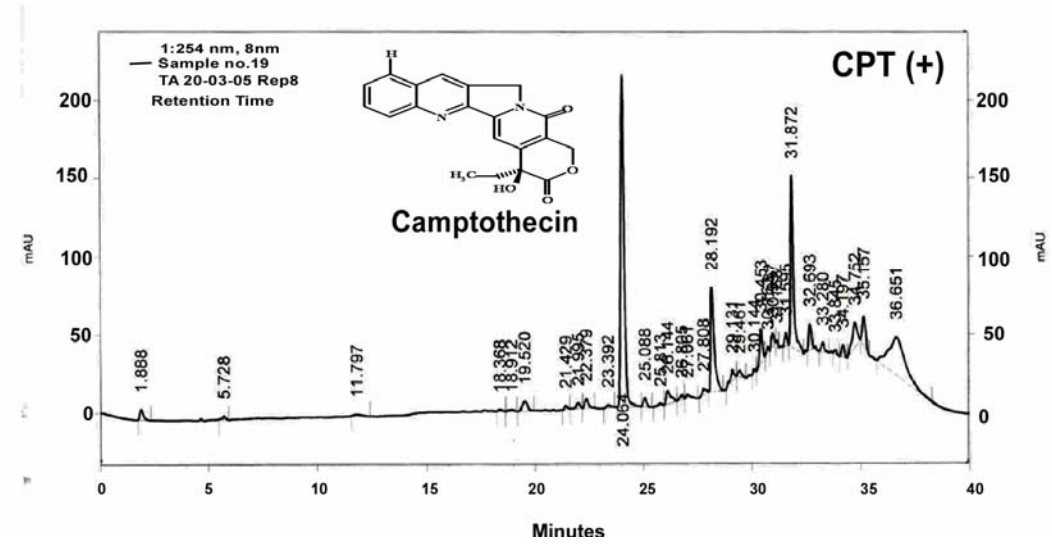

Fig. 1 f.

Fig. 1. Camptothecin (a) Identification and establishment of endophyte (b) showing dendrogram exhibiting the phylogenetic position of isolate RJMEF001 as obtained by alignment of large subunit ribosomal gene sequences (~300bp) using Clustal W and; (c) Pair-wise distance matrix of large subunit ribosomal gene sequence of different isolates showing the Phylogenetic position of strain RJMEF001 with different levels of percent divergence. HPLC based fingerprinting of CPT standard (d) at 24 min eluted using a gradient system of water and acetonitrile;λ = 254 nm. Comparative HPLC profile of CPT- fraction (e) and CPT+ fraction (f). The two fractions (e) and (f) exhibit the presence/ absence of peak at 24min respectively.

Table –I. *Growth and production kinetics of Entrophospora infrequens in shake flask (100ml)*

Incubation Time (h)	Temperature	*p*H	Mycelial dry wt. (g/lit)	CPT (µg)/ 100g dry wt.
0	28	5.6	-	-
48	29	4.0	6.91	204.25
96	29	3.8	15.81	575.50
144	30	3.0	15.45	388.70
192	29	3.2	18.11	308.75

Pro-Antioxidant Activity in Lipid Phase

The initiating product of radiation-induced free radical-mediated cascade of reactions in lipid phase is peroxyl radicals. In this assay, ferrous chloride and thiocyanate react with each other to produce ferrothiocyanate by means of hydroperoxides. The enhanced/decreased absorbance (with respect to control) indicates the prooxidant/antioxidant activity in lipid phase. As can be seen from **Fig. 2,** the pro-antioxidant activity of CPT- and CPT+ in the lipid phase was compared using ammonium thiocyanate assay. The lipid phase (linoleic acid emulsion) can undergo auto oxidation due to the presence of emulsifying agent, thereby building up the levels of peroxyl radicals. In a time-dependent study, both CPT- and CPT+ exhibited significant ($p<0.05$) pro-antioxidant activity at 50µg/ml **(Fig. 2A-2B)**. Pro-antioxidant activity was found to be significantly ($p <0.05$) higher in case of CPT+ **(Fig. 2A)**, as compared to CPT- **(Fig. 2A)**. CPT- exhibited significant ($p <0.05$) peroxyl radical scavenging activity (~antioxidant activity) at all concentrations and maximal activity was observed at 500 µg/ml (27, 48h time intervals) and beyond 500 µg/ml it becomes toxic i.e. enhancing the levels of degradation of linoleic acid **(Fig. 2A)**, indicating the ability to generate free radicals in lipid phase. The comparison of CPT+ exhibits a decrease in the antioxidant activity at corresponding concentration **(Fig. 2B)**. Supra-lethal radiation stress (0.25 KGy) induces a rapid increase in peroxyl flux in a time–dependent manner. CPT- exhibited significant ($p<0.05$) peroxyl radical scavenging activity at 250µg/ml (27h) against radiation-

induced peroxidative stress **(Fig. 2C)** and such activity was found to be decreased in case of CPT+ **(Fig. 2D)**. The prooxidant activity at 50µg/ml was maintained even in case of radiation-induced peroxidative stress **(Fig. 2C-D)**. The significantly ($p<0.05$) higher pro-antioxidant activity at 50 µg/ml (with/without radiation stress), was observed in CPT+ as compared to CPT-, which could be attributed to the presence of camptothecin **(Fig.2E and Fig.1D)**. In higher concentration range (250-500 µg/ml), the fractions exhibited a shift in antioxidant activity i.e., decrease in absorbance indicating increase in peroxyl radical scavenging activity **(Fig. 2E)**. CPT- exhibited a prominent shift in antioxidant activity as compared to CPT+, indirectly indicating that the absence of CPT enhances the antioxidant activity. It also indicated the synergistic and/ or antagonistic role of other constituents present in the fractions **(Fig.1E)**. It also indicates the pro-oxidant activity of lipid soluble bioactive constituents including camptothecin (xlogP = 1.4) extracted in the non-polar solvent system of chloroform with dielectric constant of 4.6 as compared to the active constituents (without any camptothecin) eluted using methanol with dielectric constant of 32.6. The shift in biphasic activity (in the presence/absence of radiation stress) in these two fractions directly indicates about different spectrum of bioactive constituents present in these two fractions.

As observed in **Fig.2E,** a significant prooxidant activity at 50µg/ ml of CPT+, as compared to CPT-, exhibits a significant ($p<0.05$) increase in the presence of radiation flux indicating its ability to damage cell membranes or sensitize the membranes for radiation–induced peroxidative damage. Such radio-sensitization potential has wide applications in killing cancerous cells. The results were found to be in corroboration with earlier findings of radio-sensitizing ability of camptothecin (19). Further a prominent shift to antioxidant potential at higher concentrations could be attributed to the combined action of different lipophillic constituents present in the fraction. A similar type of biphasic behavior (prooxidant-antioxidant shift) has been reported in other natural plant products like luteolin, ascorbic acid and quercetin etc. Luteolin and quercetin have been reported to have similar mode of inhibition of *Leishmania donovani* topoisomerase-I activity like that of camptothecin. Differential induction of *Leishmania donovani* bi-subunit topoisomerase I-DNA cleavage complex by selected flavones (luteolin and quercetin) and camptothecin has been reported (31). The predominant antioxidant activity of CPT- in lipid phase can be visualized as radiomodifying potential against radiation-induced membrane degeneration, as reported with other natural plant products with similar potential by virtue of their respective antioxidant abilities (7, 22).

Electron Donation Potential in Aqueous Phase

The biological system comprises of water as major solvent system for all biochemical reactions, thus net electron donation potential of the fractions was also evaluated in the aqueous phase using ferrithiocyanate assay (22). Numerous workers have reported the reductant power of different natural plant products e.g., lotus plumule and rhizome of *Podophyllum hexandrum* (7, 22, 32). They have suggested that reducing power is associated with antioxidant activity in aqueous phase. The inactivation of oxidants by reductants is a redox reaction in which free radicals get stabilized by the expense of oxidation of reducing agents as monitored in reduction of Fe^{3+}/ferricyanide complex to form ferrous form leading to the formation of Perl's Prussian blue (700nm). Such electron donation (aqueous phase) is a characteristic property and indicates total host capacity to withstand free radical stress and was found to be in positive correlation with the presence of CPT **(Fig. 3)**. Both CPT- and CPT+ exhibited dose-dependent (1-2000µg/ml) increase in absorbance, recorded at 700nm with R^2 = 0.965 and R^2 = 0.912 respectively. CPT+ was found to have higher reducing power in comparison to CPT- at all the concentrations. This observation of higher activity associated with CPT+ could be attributed to the enrichment of the sample with numerous active constituents soluble in aqueous phase. The increase in reducing power beyond 50µg/ml showed a significantly ($p<0.05$) higher activity in CPT+. The electron donation potential was evaluated in terms of end product readable at 700nm. There is a direct correlation between the electron donation potential and the antioxidant activity in aqueous phase, which explains the difference in nature of these two fractions in different types of phases. It is an overall effect of all the constituents present in the chloroform and methanol extract of the endophyte in an *in vitro* system. While on the other hand, an electron donation to Fe (III) or

Cu (II) yields redox active Fe (II) or Cu (I) metal ions that are able to amplify the free radical-mediated oxidative stress. This can directly be attributed to the presence of camptothecin (CPT) present in the CPT+fraction. Such observation can further be supported by the action of reservatrol-mediated conversion of Cu (II) to Cu(I)- induced DNA strand breaks (28). It supports the ability of fractions to exhibit a biphasic behavior in a concentration-dependant manner due to a balance between the total reducing/ scavenging ability at that concentration. The above behavior can only be understood by evaluation of free radical quenching ability of fractions.

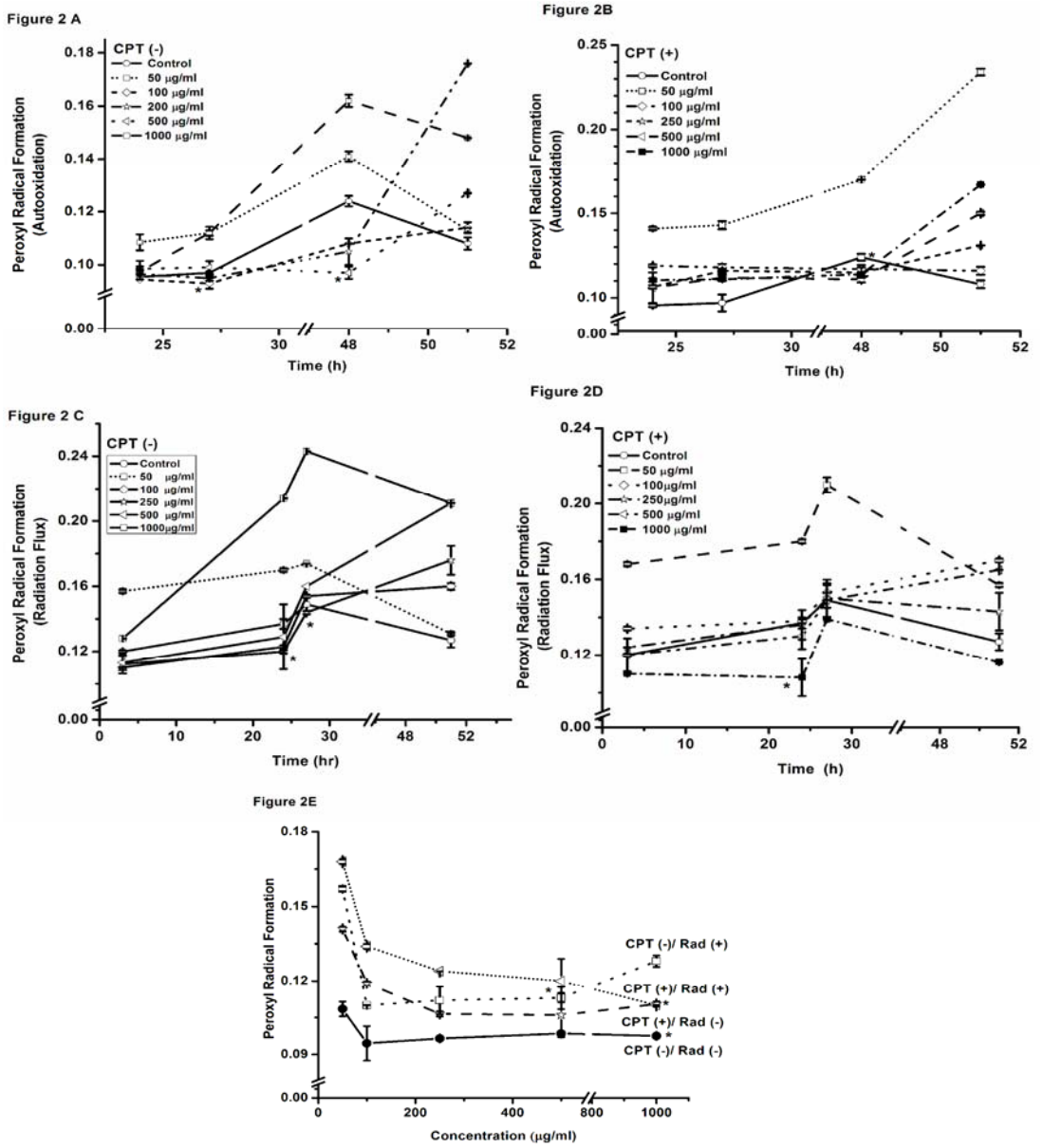

Fig. 2. Total pro- antioxidant activity (lipid phase) at different concentrations (50-1000µg/ml) of CPT(+) and CPT(-) evaluated using ferric thiocyanate method in linoleic acid system. Increase/decrease in absorbance (500nm) indicates pro-/anti-oxidant activity compared using Tukey's HSD test performed after One-Way ANOVA analysis at significant ($p<0.05$) level with respect to respective controls and other concentration groups. (A) Autooxidation of linoleic acid with emulsifier (Tween 20) and pro-antioxidant activity by CPT (-) at 24, 27, 48h [(*) indicates maximal peroxyl radical scavenging activity at 500µg/ml]; (B) Autooxidation of linoleic acid with emulsifier (Tween 20) and pro-antioxidant activity by CPT (+) at 24, 27, 48h [(*) indicates antioxidant activity from 100-1000µg/ml / prooxidant activity at 50µg/ml]; (C) Radiation-induced linoleic acid degradation (peroxyl radicals build up) in the presence of CPT (-) at 3,24,27h [(*) indicates maximal peroxyl scavenging activity at 250µg/ml]; (D) Radiation-induced linoleic acid degradation (peroxyl radicals build up) in the presence of CPT (+) at 3,24,27h [(*) indicated maximal peroxyl scavenging activity in the presence of radiation stress at 1000µg/ml+3h]; (E) Comparative evaluation of pro-antioxidant activity of CPT(+/-) against radiation (+/-) –induced oxidative stress [(*) indicates optimal antioxidant effect].

Figure 3

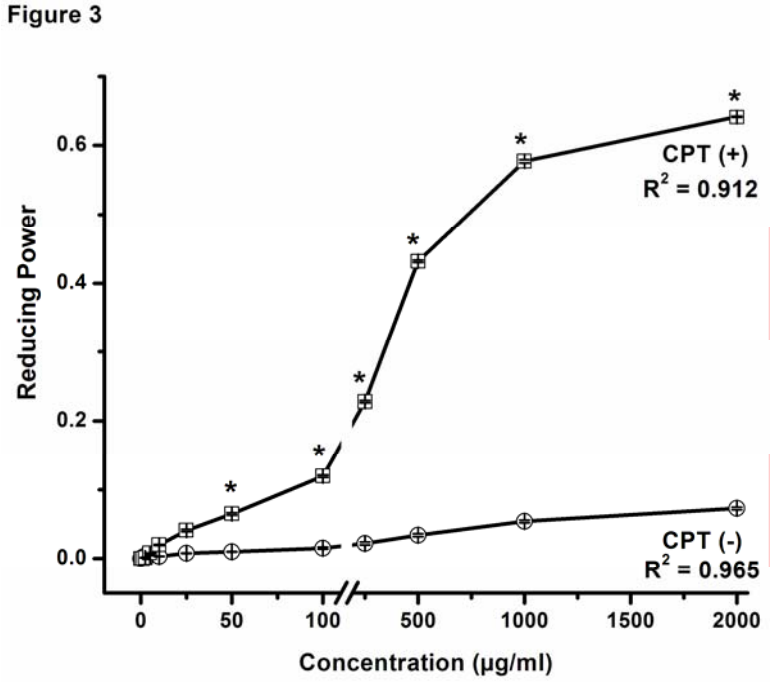

Fig. 3. Total electron donation (reducing) potential at different concentrations (1-2000µg/ml) of CPT (+) and CPT (-) evaluated using increase in absorbance (Perl Prussian blue at 700nm) with respect to concentration. (*) indicates significantly ($p<0.05$) higher reducing potential of CPT (+) as compared to CPT(-) evaluated using student's t-test.

ROS and RNS Quenching Potential

Radiation damage is induced by free radical mediated oxidative stress and the primary free radicals like superoxide radicals initiate the cascade of reactions leading to oxygen-mediated stress. Superoxide anion, a primary free radical, is generated due to the leakage of electrons from complex III of electron transport chain (ETC), as a byproduct of flavin-catalysed reaction under physiological conditions or such flux may enhance significantly in the presence of radiation-induced oxidative stress mediated by reducing of iron complexes such as cytochrome-c (33, 34). This oxygen centred radical with selective reactivity, particularly a weak oxidant, is able to generate singlet oxygen and hydroxyl radicals, which cause the peroxidation of lipids. It may cause direct lipid peroxidation too and its magnifying effects are due to amplification of redox active Fe^{2+} ions. Superoxide anions derived from dissolved oxygen by riboflavin illuminate system will reduce NBT in this system. In this method, superoxide anion reduce the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrometrically at 560nm. CPT- and CPT+, both exhibited dose-dependent (0.25-2 mg/ml) increase in super-oxide ion scavenging activity with $R^2 = 0.985$ and $R^2 = 0.963$ respectively **(Fig. 4A)**. CPT+ exhibited significantly ($p<0.05$) higher scavenging potential at all concentrations tested, as compared, to CPT-fraction which is in direct correlation with its electron donation potential in aqueous phase in the tested concentration range (250-2000µg/ml).

A number of flavonoids are reported to be antioxidant by virtue of their superoxide anions scavenging potential. The higher free radical scavenging activity of CPT+ could be attributed to the presence of CPT and other constituents **(Fig. 4A)** that are able to donate electrons to these free radical moieties converting them to stable reaction products. Such studies are in corroboration with other studies, wherein the electron donation leads to development of stable products non-interactive towards biomolecules thereby reducing radiation-induced free radical-mediated oxidative damage.

The reduction of chronic diseases, DNA damage, mutagenesis, Carcinogenesis and inhibition of bacterial growth is often association with the modulation of free radical propagation in biological systems. One such free radical is nitric oxide that exhibits a dual

behavior *in vivo* inside the body. On the one hand, it is an important signaling molecule while on the other ; macrophages use it to remove the foreign debris/ bacterial cells. The nitric oxide scavenging activity of the fractions was evaluated in terms of the percentage inhibition of formation of azo-complex, which was found to increase in a concentration dependent manner [2.5- 100 µg/ml; R^2=0.988 (CPT-) and R^2=0.947(CPT+)]. Both exhibited comparable activity between 2.5-50 µg/ml, while beyond 50µg/ml CPT- exhibited significantly ($p<0.05$) higher activity as compared to CPT+, which could be attributed to its inherent antioxidant activity **(Fig. 4B)**. However, the biphasic behavior tested in terms of end products does not explain the inherent ability of fractions to act over a period of time and thus total ferric reducing ability in plasma (FRAP) was estimated, using rutin as standard.

Figure 4A

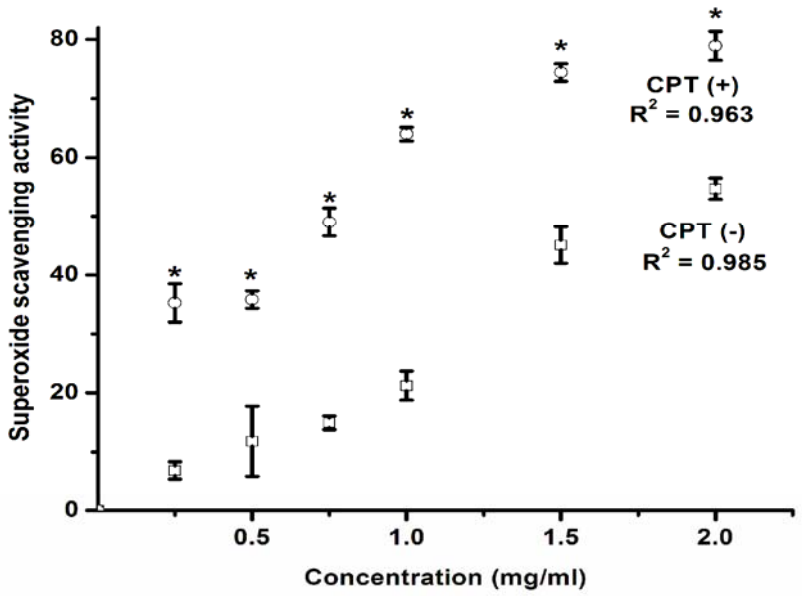

Figure 4B

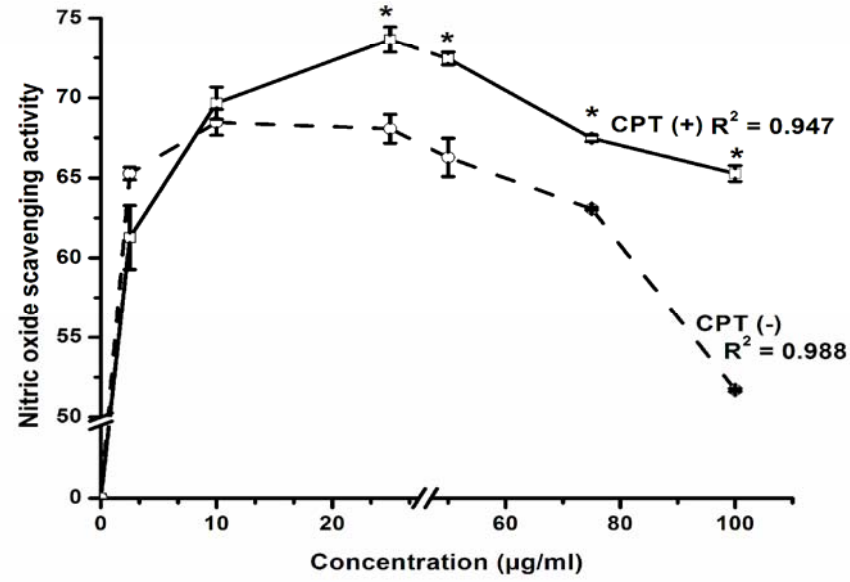

Fig. 4. Free radical scavenging potential: (A) Superoxide anion radical scavenging potential of CPT(+/-) at concentration range (0.5-2 mg/ml) evaluated using % inhibition of NBT^{2+} to formazan readable at 560nm- (*) indicates significantly (*p<0.05*) higher scavenging activity of CPT(+) as compared to CPT (-); (B) Nitric oxide scavenging activity of CPT (+/-) evaluated using % inhibition of formation of Azo dye- (*) indicates significantly (*p<0.05*) higher scavenging activity of CPT (+) as compared to CPT(-).

Ferric Reducing Ability- Kinetic Analysis

FRAP equivalence was evaluated using a micro FRAP assay. In view for comparing Ferric Reducing Ability in plasma, rutin was used as a standard. Both the fractions exhibited a dose-dependant effect evaluated over a period of 5100 seconds **(Fig. 5A-B)**. FRAP equivalence values of CPT- and CPT+ were comparable [FRAP Eq–CPT+ > FRAP Eq-CPT-:: 1.02 ± 0.04) over a period of time (time points: x, 8x and 16x wherein x=300s), indicating that there are some active constituents in CPT+ acting as antioxidant moieties predominantly in the non-lipid phase (antagonistic to the predominant action to CPT in the lipid phase as observed in linoleic acid based assay). The higher values of FRAP equivalence of CPT+ indicates its anticancer and radiosensitizing potential (biphasic activity), as compared to CPT- (antioxidant) **(Fig. 5A-B).**

Figure 5A

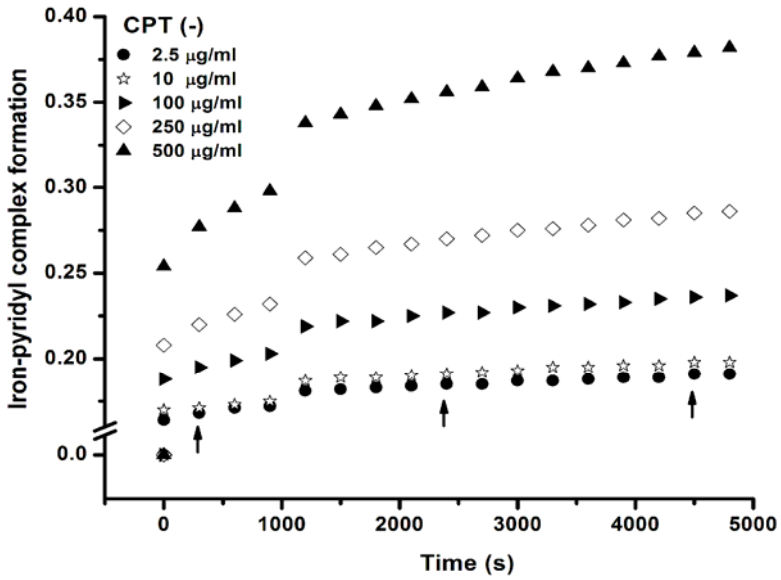

Figure 5B

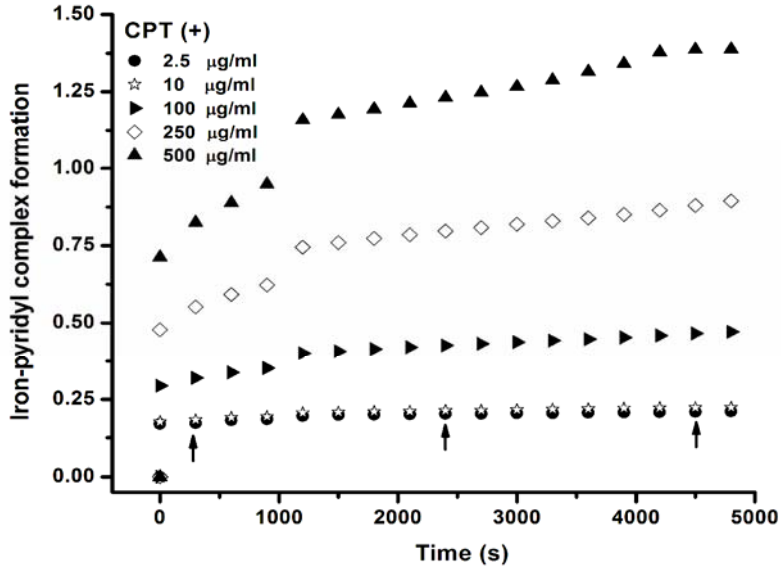

Fig. 5. Time-dependant kinetics analysis of Fe^{3+}/Fe^{2+} conversion mediated by different concentrations of (A) CPT(-) and (B) CPT (+) evaluated using modified FRAP assay for a period of 5100 seconds against rutin as standard (data of rutin not shown). A concentration-dependent increase was used to evaluate FRAP Equivalence (FRAP Eq) at x, 8x and 16x wherein x= 300s. FRAP Equivalence of CPT (+) was found to be 0.487 as compared to 0.473 [CPT (-)] with respect to rutin as standard at x=300s.

CONCLUSION

In conclusion, fractions of *Entrophospora infrequens* exhibited both pro- and anti-oxidant activities. CPT+ fraction exhibited significant ($p<0.05$) pro-oxidant behavior that could be attributed to the presence of CPT. CPT+ fraction also possesses moderate free radical scavenging potential and nitric oxide modulatory activities. CPT, present in *Camptotheca acuminata,* has been shown to possess significant anti-cancer activity ~ pro-oxidant behavior (35). In the present study, the CPT devoid fraction (i.e., CPT-) exhibited predominantly antioxidant behavior with higher free radical scavenging activity as compared to CPT+. These observations indicate that the endophyte (*Entrophosphora infrequens)* possesses potential as anti-cancer agent and has prospective utility for development of newer antioxidants, radioprotective drugs and can be utilized in a sustained manner as a prominent source of camptothecin. Wu and coworkers (1995) reported that out of 17 constituents of *Nothapodytes foetida* identified, only 3 are significantly cytotoxic (36). It is possible that the endophyte synthesizes the non-cytotoxic constituents similar to those present in *Nothapodytes foetida,* and these may be imparting the CPT- fraction its antioxidant activity. Further studies are needed to corroborate these findings to explore the potential of other active constituents in radioprotection (by virtue of their antioxidant behavior). Based on the discussion above, the studies clearly indicate the inherent radiomodifying potential of the fractions. CPT (+) could be utilized as an anticancer drug or a potent radiosensitizer, while CPT (-) fraction can be explored to identify potent radioprotective molecules or antioxidant moieties, which should be useful in the clinical management of different types of reactive oxygen species (ROS)- mediated disorders.

ACKNOWLEDGEMENTS

The authors are grateful to Defence Research Development Organisation, Ministry of Defence, Delhi and Institute of Integrative Medicine (formerly Regional Research Laboratory, Jammu) for providing research/laboratory facilities. Drs. SC Puri and T. Amna gratefully acknowledge the support of Dr. Vijeshwar Verma in phylogenetic analysis carried out using CLUSTAL W and guidance and support of Dr. GN Qazi.

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