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## Current Trends in Biotechnology and Pharmacy

**ISSN 0973-8916**

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Transcriptional and translational leakiness in expression of a fungal cyclopropane fatty acid synthase in the <i>Escherichia coli</i> pET-vector expression system

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Abstract
We expressed the putative cyclopropane synthase Cfs1 from the mushroom <i>Coprinopsis cinerea</i> and the native bacterial Cfa enzyme in <i>Escherichia coli</i> as fusion proteins with an N-terminal 10xHis-tag and a proteolytic Factor Xa site, using the commercial pET-16b vector with the T7lac promoter for expression. Proteomics detected both proteins with high Mascot scores via trypsin-digested peptides in total cytoplasmic protein samples from transformed BL21(DE3) bacteria, induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) for T7 RNA polymerase production from the T7 phage gene 1 present in the host genome under control by the lacUV5 promoter. In lower amounts, Cfs1 and Cfa were also found in protein samples from non-induced cells based on the known transcriptional leakiness of the pET-system in expression of T7 RNA polymerase. Proteomics detected both proteins with high Mascot scores via trypsin-digested peptides in total cytoplasmic protein samples from transformed BL21(DE3) bacteria, induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) for T7 RNA polymerase production from the T7 phage gene 1 present in the host genome under control by the lacUV5 promoter. In lower amounts, Cfs1 and Cfa were also found in protein samples from non-induced cells based on the known transcriptional leakiness of the pET-system in expression of T7 RNA polymerase. As a novelty, the results on Cfs1 from proteomics suggest that the sequence at the pET-16b-cfs1 fusion point TCTCGAGGT CCTATG (BamHI cloning site in italic, native cfs1 ATG start codon in bold) serves in transcripts as a minor RBS (ribosome binding site) to inadvertently initiate protein synthesis on the native cfs1 start codon. The first peptide MPAHHHPSSAPCVSSFPSSS of the native Cfs1 protein was found in E. coli which is only possible if the normal cfs1 ATG was recognized as the start codon in protein biosynthesis. The GAGG-stretch in the above DNA sequence with a 7-bases-aligned spacing to the native cfs1 start codon features a partial Shine-Dalgarno (SD) sequence.

Key words: pET-vector, heterologous protein expression, basal transcription, translational leakiness, Shine Dalgarno (SD) sequence.

Introduction
<i>Coprinopsis cinerea</i> is an edible mushroom that is regularly used as a model species in research on Agaricomycetes (1). Several mutants in fruiting body development were analyzed to identify gene functions required for the fruiting process. Among, gene cfs1 for a potential cyclopropane fatty acid (CFA) synthase was found to be essential for fruiting. When defective, the fungal mycelium on the contrary grows still normal (2). In the grown mycelial cultures of the wildtype, microscopic primary hyphal knots with multiple short stunted side branches are formed in the dark by localized intense hyphal branching. When further cultured in the dark, primary hyphal knots can transform into multicellular sclerotia which are compact round resting structures with an outer brown rind (3,4). Alternatively when receiving a light signal, the highly branched primary hyphal knots in wildtype strains can enter the fruiting pathway and convert into compact secondary hyphal knots (5,6) in which mushroom...
cap and stipe tissues will differentiate (6,7). However, a cfs1 mutant is unable to perform this light-induced step of secondary hyphal knot formation, unless the gene defect has been complemented with a wildtype gene copy via transformation (2). In accordance, expression of the cfs1 gene is also light-induced, together with secondary hyphal knot formation and it continues in further development (2,5).

CFA synthases are known from many bacteria (8-10). Of these, the Escherichia coli enzyme Cfa is best characterized (11-16). CFA synthases, in full enzymatic name cyclopropane-fatty-acyl-phospholipid synthases (EC 2.1.1.19), catalyze the post-synthetic transfer of a methylene group from S-adenosyl methionine (SAM) across cis-double bonds in mono-unsaturated fatty acyl chains in phospholipids, thereby replacing the double bond with a methylene bridge to generate a CFA (8-16). More specifically, E. coli Cfa cyclopropanizes unsaturated double bonds in C16:1 (9) and C18:1(9) fatty acid chains (in palmitoleic and cis-vaccenic acid, respectively) in membrane-localized phospholipids at the \( \Delta_9 \) positions. Thereby, 17:0cyclo and 19:0cyclo CFAs are yielded (8,9,11-15,17), while other enzymes may only form 17:0cyclo CFAs (9). Cyclopropanation of unsaturated fatty acids (UFAs) alters membrane fluidity, permeability and integrity stability. This provides resistances to cells to withstand multiple physical and chemical environmental stressors such as acids, high salt, low or high temperature, and others (8,15,18-24). Manifold interests in CFAs exist then in applications in biotechnology and medicine. CFAs might be exploited in stress responses to improve microbial growth in fermentations in presence of inhibitors and under acid stress (25-27), and for better survival of probiotics (28,29). CFAs can also be target for antibiotic drugs designed against pathogens (30,31). As ingredients, they may increase the value of edible oils and of health-promoting food (32,33). Further, they serve in production of feed stocks for biofuels and lubricants (34,35).

Certain protists (30,36,37), fungi (2,38) and plants (39-42) also have genes for Cfa-like enzymes and in some instances, developmental defects by mutations have been recorded (2,37,42). Leishmania and plant enzymes have been reported to undergo similar cyclopropanation reactions than E. coli Cfa, but in part on other fatty acids (36,37). Of fungi, no such enzymatic reaction has yet been shown by experiments. In this study, we expressed C. cinerea Cfs1 as a 10x His-tag-fusion protein under control of a T7-lac promoter in E. coli, using the native cfa gene in parallel as a control gene and making use of the commercial E. coli pET-vector expression system (43). The Cfs1 protein was abundantly expressed in the E. coli B strain BL21(DE3), under isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) induction of the chromosomally inserted T7 RNA polymerase gene controlled by the efficient lac UV5 promoter. However, we also observed protein expression under non-inducing conditions and, surprisingly, expression of unfused Cfs1 suggesting coincidental translation on the transcripts from the native ATG start codon cloned downstream to a cryptic ribosome binding site (RBS). Our work reveals therefore types of leakiness in the pET system at both levels, transcription and translation.

Materials and methods

Vector constructs: As formerly reported (44,45), full-length cDNA of the C. cinerea wildtype gene cfs1 subcloned in vector pBluescript KS(-) in E. coli DH5\( \alpha \) was PCR-amplified with primers PETcfs1 (5' TGATA GGTACC TATG CCGGCC CACCAC 3') and M13 forward(-20) (5' GTAAAACG ACGGCCAG 3'), and the genomic sequence of the functional E. coli cfa gene (GenBank NC_000913) with primers Bam.cfaF (5' CAC CGACCAGTGATGGAGAAACTG GATCCGATG AGTTCATCGTGATATAG 3´) and Bam.cfaR (5' CTTG GCAGCAGCTAGATTGGATGATCACA ATGAAATGATCCAC 3´). BamHI restriction sites are in italic, native start codons in bold. Amplified BamHI-digested fragments were subcloned into the BamHI site of the expression
vector pET-16b. Cloning in sense direction in frame with the sequence for the 10xHis-tag (Fig. 1) resulted in sense vectors pET-16cfs1 and pET-16bcfa. Cloning in antisense direction gave antisense vectors pET-16cfs1-inv and pET-16bcfa-inv. All constructs were transformed into the Lon- and OmpT protease-deficient E. coli B strain BL21(DE3) with a genomic lacUV5 promoter-controlled T7 RNA polymerase gene as functional strain for expression in the pET-system (43). All constructs were further transformed into the protease-proficient E. coli K12 wildtype strain ZK126 and its mutant YYC1272 (cfa:kan of ZK126) with an inactivated cfa gene (46).

Protein production: Selected BL21(DE3) clones were cultivated for 2.5 h in 3 ml LB/amp medium (100 mg ampicillin/l LB) at 37 °C and 200 rpm to an OD600 of 0.2. Cells were transferred into 100 ml LB/amp in 500 ml Erlenmeyer flasks for 3 h growth at 37 °C and 200 rpm until an OD600 of ≥0.5. Cultures were split into two, with 50 ml LB/amp each in 100 ml Erlenmeyer flasks. One culture was induced by 1 mM IPTG as described in the pET-vector manual (43) and the other remained non-induced during further growth of all cultures for 2 h at 37 °C and 200 rpm to an OD600 of ≥1.0. Flasks were then placed onto ice for 5 min. Each 1 ml of cells was harvested from well-mixed cultures and centrifuged. Supernatants were discharged and the pellets drained by tapping the inversed tubes onto a paper towel to remove excess medium. Pellets were completely resuspended by mixing in 100 μl of 1x phosphate-buffered saline (PBS). Then, for lysis of cells, 100 μl of 4x SDS (sodium dodecyl sulfate) sample buffer (250 mM Tris-HCl pH 6.8, 6% SDS, 300 mM DTT (dithiothreitol), 30% glycerol, and 0.02% Bromophenol blue; Cat. No. 70607-3; Novagen, Darmstadt, Germany) was added to a sample and sonicated with a microtip until the pellet was completely dissolved. Immediately after, samples were heated for 3 min at 85°C to denature the proteins and then stored at −20°C until further use (47).

For total cytoplasmic protein isolation from acid-stressed ZK126 and YYC1272 clones, strains were cultured and incubated at pH 3.0 and pH 7.0 in acid tests as described before (46). Single colonies were picked from freshly streaked LB/amp agar plates and inoculated into 25 ml LB/amp in 100 ml Erlenmeyer flasks to grow overnight at 37 °C and 200 rpm on a shaker to reach an OD600 of 2.0. Four ml of the precultures were then transferred into 25 ml LB/amp in 250 ml Erlenmeyer flasks (4 ml preculture corresponded to an OD600 of 0.3 to 0.4 in the main culture) and incubated at 37 °C for 3 h at 200 rpm on a shaker to reach an OD600 of about 1.2 for the actual acid test of the main culture. One ml aliquots per test sample were centrifuged for 10 s at full speed and supernatants were discarded. Pellets were washed one time with LB medium (pH 7.0). One ml LB/amp at appropriate pH (control cells at pH 7.0; acid shock cells at pH 3.0 adjusted by 6N HCl) was added per sample for 1 h incubation of resuspended cells on a shaker at 200 rpm and 37 °C. Then, proteins were isolated from the samples as described above.

Protein gel electrophoresis: SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed in a Multigel-Long chamber (Biometra GmbH, Göttingen, Germany) using 4 % stacking and 12 % resolving gels as described (47). Per well, 25 or 30 μl protein samples were loaded into the gels. Protein size marker #SM0431 from Fermentas (Vilnius, Lithuania) was used. Electrophoresis was first conducted at a constant current of 15 mA until the samples reached the resolving gel and then at 25 mA for migration of proteins into the resolving gels. Afterwards, gels were fixed in 12 % trichloroacetic acid (TCA) for at least 1 h, stained overnight in colloidal Coomassie solution (10 % phosphoric acid (v/v), 10 % ammonium sulfate (w/v), 0.12 % Coomassie Brilliant Blue G250 (w/v; Serva, Heidelberg, Germany) in a 4:1 water/methanol solution). Gels were washed with water until an optimal contrast for bands was achieved.
Protein digests, mass spectrometry and Mascot protein identification. Bands of interest were cut out of gels with a razor blade and proteins within gel pieces were trypsin-digested as described before (48). Tryptic peptides were dissolved in 2% formic acid and separated on a 12 cm capillary column (ID 180 μm) packed with 3 μm particles of Reprosil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch, Germany). Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI–MS/MS) spectra were acquired using an LC 1100 series high performance liquid chromatograph (Agilent, Waldbronn, Germany) coupled to an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Resulting raw data were analyzed with Data-Analysis v. 3.0 software (Bruker Daltonik GmbH). Proteins were identified by searching against a database of the annotated genome of C. cinerea Okayama 7 #130 combined with the SwissProt database (http://us.expasy.org/sprot/) and against NCBIProt, using Mascot software (www.matrixscience.com) and as settings C-carbamidomethylation as fixed modification, M-oxidation as variable modification, peptide mass tolerance 1.2 Da, peptide charge 1+, 2+, and 3+, MS/MS tolerance 0.6 Da, and 1 missed cleavage allowed (49). Molecular weights of pure amino acid chains were calculated with ExPaSy Compute pi/Mw (https://web.expasy.org/compute_pi/).

Results and discussion. The structure of the fusion proteins. In-frame-cloning of the complete cfa coding sequence of E. coli and of the full length cfs1 cDNA from C. cinerea into the BamHI-site of pET-16b should result in fusion proteins which are 26 aa (amino acids) longer (Fig. 1) and by 3.21 kDa heavier compared to the native proteins Cfa (382 aa; theoretically calculated molecular mass of 43.94 kDa) and Cfs1 (488 aa; theoretically calculated molecular mass of 54.4 kDa). The extra N-terminally added aa present a 10xHis-tag for protein purification over Ni-NTA columns (43).
Factor Xa site IEGR for proteolytic cleavage of the N-terminus after residue R (43) and the sequence HMLEDP resulting from translation of the vector sites in series for the restriction enzymes NdeI, XhoI and BamHI, respectively (Fig. 1). The subsequent sequences in the fusion proteins are then those of the native enzymes, continuing protein translation from the normal ATG start codon of the genes (marked in Fig. 1 for both sense-constructs).

Protein identification after expression in E. coli B strain BL21(DE3). Vector-transformed E. coli BL21(DE3) clones were cultivated for protein isolation as described in the methods. Aliquots of all protein samples were separated by SDS-PAGE and stained by Coomassie (Fig. 2). Intensities of most protein bands in the gel were comparable between the different probes, including in the samples of clones that were transformed with the antisense constructs. In the samples with the sense-plasmids pET-16bcfa and pET-16bcsf1 however, stronger blue bands were clearly visible at gel positions of around 45 kDa and 55 kDa, respectively. A slightly stronger band of corresponding size was also detectable by eye in the IPTG-non-induced sample of BL21(DE3) with the pET-16bcfa plasmid but not in the sample of BL21(DE3) with the pET-16bcsf1 plasmid (Fig. 2). The theoretical masses of the native E. coli protein Cfa and the native C. cinerea protein Cfs1 corresponded roughly to the size of the two strong bands seen in Fig. 2 in the sense-vector samples induced by IPTG. The fusion proteins produced recombinantly from the pET-16b vector should, however, be slightly larger than the native proteins by the extra 3.21 kDa obtained through the N-terminal addition of the artificial sequence MGHHHHHHHHHHHHHHHHHHHGGR (see Fig. 1).

The strong bands in the IPTG-induced samples and bands of corresponding regions from the non-induced samples were cut out of the gel and trypsin-digested for ESI-MS/MS analysis. Mascot-searches then identified proteins Cfs1 and Cfa with very high scores (768 and 296) and protein coverages (47 and 37 %) in the IPTG-induced samples but also, among other well-expressed E. coli proteins, with good scores (113 and 114) and protein coverages of 11 and 27 % in the non-induced samples (Table 1). The proteomics data thus clearly documented the successful expression of the heterologous C. cinerea protein Cfs1 from the fungal gene cloned in E. coli. Cfa outperformed other similar-sized proteins in abundant expression in the IPTG-induced pET-16bcfa sample harvested at a later logarithmic growth and was highly expressed in the non-induced sample as well (see Mascot scores and peptide numbers of best proteins in Table 1). It is of relevance that E. coli strain BL21(DE3) possesses a native cfa gene. Typically in E. coli cultivation, cfa is moderately expressed with short-lived protein products not before a strain enters...
stationary growth phases (50,51). Finding very high amounts of Cfa here in the BL21(DE3) samples suggests that the protein expression derived from the cloned cfa gene.

Detection of Cfs1 and Cfa in non-induced cells indicates leakiness in the pET-system in expression from the T7/lac promoter. Similar observations of T7/lac promoter leakiness had been reported before (43,52-57). According to the literature, leakiness in transcription depends on the E. coli strain, whether it has a T7 RNA polymerase gene and how well expression of the polymerase is suppressed (57). In the artificial pET-16b-system, induced expression of the T7 RNA polymerase gene is controlled by the lacUV5 promoter via IPTG addition, but this promoter has also some basal activity in absence of IPTG (43).

**Expression without a T7 RNA polymerase in E. coli K12 strains ZK126 and YYC1272:** All sense and anti-sense pET-constructs were also transformed into wildtype E. coli ZK126 and the cfa-mutant strain YYC1272, respectively. Cultures were grown at 37 °C as described in the method section and acid-challenged for 1 h. Then, intracellular proteins were isolated from harvested cells and separated on 12 % SDS-PAGE gels. Regardless of the E. coli strain, the transformed vector used, or the pH applied to the cultures, banding patterns in all samples were very similar (Fig. 3A and B). Bands of sizes of about 45 kDa and 55 kDa were excised from the gels, trypsin-digested and resulting peptides separated in mass-spec analysis for protein identification.

Mascot searches identified for the 45 kDa gel regions from the E. coli cfa mutant strain YYC1272 with good scores and at least two peptides per protein repeatedly: phosphopyruvate hydratase/enolase (mostly at pH 3.0), glutamate decarboxylase, elongation factor Tu (as in BL21(DE3); Table 1), isocitrate lyase (as in BL21(DE3); Table 1), NADP-dependent 6-phosphogluconate dehydrogenase (mostly at pH 3.0), and phosphoglycerate kinase pgk (mostly at pH 7.0), and citrate synthase (mostly at pH 7.0; as in BL21 (DE3); Table 1), Proteins identified from the 55 kDa mass range included 60 kDa chaperone 1 GroEL, periplasmic oligopeptide-binding protein OppA (as in BL21 (DE3); Table 1), adhesin Ag43 (mostly at pH 3.0), PTS N-acetyl glucosamine transporter subunit IIA/B/C (mostly at pH 3.0), nitrate reductase subunit beta (mostly at pH 3.0), trigger factor (mostly at pH 7.0),

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![Figure 3](image_url)

**Fig. 3.** 12 % SDS-PAGE gels of protein extracts from A. E. coli ZK126 and B. YYC1272 cells transformed with different pET-16b expression plasmids as indicated above the lanes. Protein extracts (25 μl each) from cultures were incubated either at pH 3.0 (left) or at pH 7.0 (right).
pyruvate kinase I (mostly at pH 3.0), trehalose-6-phosphate hydrolase TreC (mostly at pH 3.0), glyceraldehyde-3-phosphate dehydrogenase A, malate synthase A, and long-chain-fatty acid transporter FadL (mostly at pH 7.0). Several of these proteins in similar patterns of distribution were also detected in the corresponding samples taken from wildtype strain \textit{E. coli} ZK126 cultures. However, neither \textit{E. coli} Cfa nor \textit{C. cinerea} Cfs1 peptides were detected in any of the bands excised from the two strains.

Since wildtype strain \textit{E. coli} ZK126 contains a functional native \textit{cfa} gene, it could actually have been expected to find the native acid-induced Cfa protein at least in the samples of the acid-stressed clones of this strain (46). While a range of highly expressed proteins was found in proteomics analysis of gel-excised bands, the sensitivity in detecting tryptic peptides from the bands might not have been sufficient enough to discover poorly expressed and very instable proteins. In the literature, it is reported that the \textit{E. coli} Cfa protein is very short-lived and unstable in strain ZK126 (51). The same is probably true for Cfs1. Loos (44) formerly analyzed protein extracts from YYC1272 pET-16b\textit{cfs1} transformed cells in western blots with tetra-His-antibodies that recognize the 10x His-tag of pET-16b vector-encoded fusion proteins. She detected no band of the full-length Cfs1-fusion protein in SDS-PAGE separated protein extracts. Depending on IPTG, however, ladders of weak signals of smaller bands were found which indicated rapid breakdown of expressed 10xHis-tagged Cfs1 protein. In the experimental approach pursued here with a focus on bands of full-length protein sizes, any shorter degradation products of the 10xHis-tagged Cfs1 protein would not have been possible to discover.

Breakdown products of the Cfs1 fusion protein detected by western analysis in \textit{E. coli} YYC1272 in the absence of a T7 RNA polymerase gene proved leaky expression of Cfs1 in the strain (44). As an additional effect in the \textit{E. coli} pET-16b vector expression system, the addition of IPTG removes the blockage of transcription by the LacI repressor bound to the lac operator directly downstream to the T7 promoter (Fig. 1), (43). The leaky expression of Cfs1 in \textit{E. coli} YYC1272 then probably resulted from leaky transcription of the cloned \textit{cfs1} gene from a pseudo-promoter for the normal \textit{E. coli} RNA polymerase, mediated by some unidentified vector sequences upstream of the lac operator (44).

\textbf{Translational leakiness bases on a cryptic SD sequence overlapping the BamHI cloning site in the pET-16b vector:} Fusion proteins produced from transcripts from the pET-vectors should all contain the N-terminally added artificial sequence MGHHHHHHHHHSSFHIIEGRHML EDP (Fig. 1). In protein digests with trypsin that cuts after R or K residues unless a proline follows (58), the 20 amino acid-long peptide MGHHHH HHHHHHSSFHIIEGR might thus arise from the N-terminal fusion proteins. The short extra peptide sequence HMLEDPS in contrast should remain N-terminally linked to M as the first native residue of the protein chosen for expression (Fig. 1). Accordingly, a peptide starting with the first native M should not be present in trypsin-digests of fused proteins encoded through cloning by pET-16b, unless the native start codon is somehow also recognized for translation by error. Indeed, we unexpectedly found the first peptide MPAHHHP SSSACVSPSSSK of trypsin-digested Cfs1 in the gel-excised band from IPTG-stimulated B21(DE3) cells (Table 1, case ‘55 kDa band from IPTG-induced pET-16b\textit{cfs1} transformed cells’), in a form as it can only come from an N-terminally non-fused Cfs1 protein.

In bacteria, ribosomes bind to specific recognition sequences, the Shine-Dalgarno (SD) motif (consensus \textit{E. coli} AGGAGGU and motif GAGG in \textit{E. coli} virus T4 early genes), on mRNAs and then start translation at an ATG in average seven bases downstream to the RBS (59-61). This prompted us to search for a potential SD sequence in plasmid pET-16b\textit{cfs1} in close vicinity upstream to the natural \textit{cfs1} ATG start codon. In fact, the sequence at the pET-16b-\textit{cfs1} fusion point CTCGAGGATCCTATG (the BamHI site used in
### Table 1: Mascot analysis of trypsin-digested protein bands cut from the gel shown in Fig. 2 with proteins isolated from recombinant E. coli B21 DE3 cells

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<td>Score 259</td>
<td>Mass 44860</td>
<td>Matches 20 (12)</td>
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<tr>
<td>2 Cfa</td>
<td>Score 114</td>
<td>Mass 44337</td>
<td>Matches 25 (12)</td>
</tr>
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<td>3 Citrate synthase</td>
<td>Score 68</td>
<td>Mass 48383</td>
<td>Matches 2 (2)</td>
</tr>
<tr>
<td>4 Succinyl-CoA ligase</td>
<td>Score 49</td>
<td>Mass 44652</td>
<td>Matches 2 (1)</td>
</tr>
</tbody>
</table>

**55 kDa band from non-induced pET-16b(f) transformed cells**

<table>
<thead>
<tr>
<th>Hit Parameter</th>
<th>Protein</th>
<th>Mascot Parameter</th>
<th>Sequence with peptide hits (marked in yellow)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Tryptophanase</td>
<td>Score 121</td>
<td>Mass 33139</td>
<td>Matches 13 (8)</td>
</tr>
<tr>
<td>2 ATP synthase subunit beta</td>
<td>Score 113</td>
<td>Mass 50308</td>
<td>Matches 10 (5)</td>
</tr>
</tbody>
</table>

* Consecutive peptides for clear distinction are alternately shown in normal and in italic letters, respectively. Overlapping peptides of different length are underlined.

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Transcriptional and translational leakiness in expression in the pET-vector system
cloning is shown in italic; the native \textit{cfs1} ATG start codon in bold; Fig. 1) contains a potential erroneous RBS. Within this sequence, there is a GAGG-stretch with a 7-bases-aligned spacing to the native \textit{cfs1} start codon (as defined by alignment with the CCUCC sequence at the 3´end of the 16S rRNA (60)) which resembles a partial SD sequence. We conclude that it is this sequence that apparently can serve as a minor RBS in transcripts generated from pET-16b\textit{cfs1}.

Translation from a downstream cryptic RBS in cloned sequences of a range of eukaryotic genes has several times been reported (62-68). As much as we deduce from searches in the literature, this work gives for the first time evidence for a cryptic RBS located upstream to a cloned eukaryotic gene in the pET-16b vector sequence.

\textbf{Conclusions}

In this study, the essential \textit{C. cinerea} fruiting protein Cfs1 and the native \textit{E. coli} Cfa protein have successfully been overexpressed in \textit{E. coli} BL21(DE3) under IPTG induction from fusion genes cloned behind the T7\textit{lac} promoter in the expression vector pET-16b. We further confirmed previously described transcriptional leakiness of the pET-16b expression system based on some basal transcription of the T7 RNA polymerase gene in absence of IPTG. This resulted in still good Cfs1 and Cfa expression from the fusion genes in pET-16b in absence of IPTG. Another type of transcriptional leakiness in \textit{cfs1} expression occurred in \textit{E. coli} YYC1272 in the presence of IPTG and the absence of T7 RNA polymerase by an unknown upstream pseudo-promoter in pET-16b for the normal \textit{E. coli} RNA polymerase (44). To our best knowledge newly in this study, we found evidence for leakiness also in translation, mediated by a cryptic SD sequence within the polylinker of the pET-16b vector and directed towards production of the unfused Cfs1 protein from its native start codon.

\textbf{Acknowledgements}

We are grateful to Dr. Andrzej Majcherczyk for technical introduction to Mascot analysis.

\begin{thebibliography}{9}
\end{thebibliography}


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49. Rühl, M., Majcherczyk, A., Kües, U. (2013). Lcc1 and Lcc5 are the main laccases secreted in liquid cultures of *Coprinopsis cinerea* strains. Antonie van Leeuwenhoek 103:1029-1039


Transcriptional and translational leakiness in expression in the pET-vector system
Abstract

Foot and mouth disease (FMD) is highly infectious, contractable disease of ungulates and is associated with cattle which causes high economic losses in livestock industry. Virtual control of FMD requires specific and sensitive point-of-care diagnostic tools to eradicate the disease spreading. So far, the diagnostic tools used for Foot and Mouth Disease Virus (FMDV) are molecular based assays which are more expensive. The main objective of this research study is to develop and evaluate point-of-care test for rapid detection of FMDV in animals. For this study, a highly specific and sensitive FMD non-structural protein (NSP) antibody rapid immunodiagnostic assay was developed using recombinant 3ABC (r3ABC) protein of FMDV for the detection of antibodies against FMDV and compared with commercial ELISA. The FMDV 3ABC gene was cloned into pET28a (+) vector and the gene product was expressed in E. coli BL21 cells. The expressed r3ABC protein was detected by SDS-PAGE analysis which resulted in a protein band with approximate molecular weight of 60 kDa. Purified r3ABC antigen was used as a detection reagent in rapid Lateral Flow assay (LFA). The diagnostic Assay was performed with 33 reference and 380 field samples and results showed that 94% sensitivity and 98.9% specificity. This study revealed that successful expression of 3ABC gene and diagnostic assay development lead to the identification of diseased animals. It further demonstrated that LFA as potential diagnostic tool for the point-of-care diagnosis of FMDV in large herds within limited time.

Key words: Foot and Mouth Disease, Sensitivity, Specificity, Lateral Flow Assay, ELISA, Point-of-Care.

Introduction

Foot and Mouth Disease (FMD) is the most economically important disease of animals and endemic in majority of the Asian countries. FMD is a highly contagious viral disease in livestock and has serious economic impact in cloven footed animals. The disease spreads in cattle, buffaloes, goat, swine and more than 70 species including domestic and wild animals (1). FMD's causative
agent is Foot and Mouth disease virus (FMDV) and it belongs to the family *Picornaviridae* and genus *Aphthovirus*. FMD virus exists as seven immunologically distinct serotypes O, A, C, Asia 1 and SAT 1, SAT 2, SAT 3, (Southern African Territories) and multiple subtypes in each serotype (2,3). All these serotypes are recorded so far in India since 1995 onwards whereas serotype C was not reported in India (4,5). Due to the infectious nature, FMD is classified as a list “A” disease by the World Organization for Animal Health (OIE) (FAO/OIE/WHO (1995) (6). FMD causes the lameness and vesicular lesion on tongue, teats, feet, besides huge weight loss and milk reduction. Many developed and undeveloped countries obtained FMD free status by making mass vaccination and by strict trade policy. FMDV is having RNA as genetic material and encodes various structural and non-structural proteins (7). Mostly, inactivated FMD whole virus vaccines were used for prevention of disease all over the world. This form of vaccine consists of killed viral particles and are supposed to evoke antibodies against the structural proteins of FMDV. Majority of the vaccine makers remove NSPs while producing the vaccines and animals probably produce antibodies to non-structural proteins (NSPs) (8,9,10). The animals naturally infected with the FMD virus are supposed to express NSP proteins and elicit the immune response that can be detected using a diagnostic prospective. These particular approaches of diagnostic methods are meant to differentiate the infected animals from vaccinated herds. Majority of the testing methods used for the detection of FMD infected animals were produced by a combination of NSPs 2A, 2B, 2C, 3A, 3B, 3AB, 3D and 3ABC which in-turn used to develop immuno diagnostic assays. Among all NSPs, 3ABC protein is highly immunogenic and described (11) as one with high amount of antibodies against 3ABC antigen in animal sera. Currently FMD serological diagnosis was carried out using Complement Fixation Test (CFT) and Enzyme Linked Immunosorbent Assays (ELISAs), but these assays need more time to execute and trained laboratory personnel. But, screening large number of animals in less time at field level requires point-of-care tests. At present, lateral flow assays have more importance for detection of pathogenic antigen in clinical specimens.

The main objective of the present study is production of FMDV recombinant 3ABC antigen using a bacterial expression system and evaluation of lateral flow immuno-chromatographic assay using FMDV NSPs (r3ABC) to determine the diagnostic efficacy of lateral flow immunoassay.

**Materials and Methods**

**Production of recombinant 3abc protein of FMDV:** A 1326 bp long fragment whole 3ABC gene (from the viral nucleic acid at the repository of Genomix CARL) was cloned into pET-28a(+) vector and transformed into *E.coli* BL21 competent cells as per standard protocol (12) and grown on Luria Bertani (LB) agar plate containing 100 mg/ml kanamycin. For the expression and purification of proteins from *E.coli* BL21 cells containing pET-28a (+)/3ABC, construct was cultured overnight at 37°C in 5ml LB broth containing 100 mg/ml kanamycin. The overnight culture was transferred into 250 ml fresh LB kanamycin medium and allowed to grow till the OD reaches 0.7 to 0.8. The cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h. After the cells were harvested, the cell pellets were lysed in lysis buffer (8 M urea, 0.1M NaH2PO4, 10 mM Tris-HCl, pH 8.0) and the suspension was sonicated. Then the lysate was passed through the column. The column was washed with 10 volumes of wash buffer (8 M Urea, 0.1 M NaH2PO4, 10 mM Tris-HCl, pH 6.3 ) and the protein was eluted with elution buffer (8 M urea, 0.1 M NaH2PO4, 10 mM Tris-HCl, pH 4.5) in fractions of 500 ml. Purified protein concentrations were determined by Bradford method and protein Anil et al
concentrations were analyzed with SDS-PAGE gel electrophoresis followed by Coomassie brilliant blue staining. Protein fractions were aliquoted and stored at -80°C till further use.

**Sample collection and study area:** The present study was majorly conducted at the Genomix CARL Pvt.Ltd., (Pulivendula, AP, India), Dodla Dairy (Pulivendula, AP,India) and Genomix Molecular Diagnostics Pvt. Ltd., (Hyderabad, India). The third-party evaluation study was carried out at the Department of Veterinary Public Health and Epidemiology, P.V.N.R. Telangana Veterinary University, Hyderabad. Analysis of the study was carried out in 180 vaccinated bovine serum samples, 200 non-vaccinated bovine serum samples and 33 positive and 20 negative reference serum samples. The positive and negative reference serum samples were provided by P.V.N.R. Telangana Veterinary University, Hyderabad. Blood samples of approximately 6 ml volumes were drawn from jugular vein of each animal in plain vacationer tubes (BD) and serum was collected from the tubes after clotting the blood by centrifuging the tubes at 4000 x g for 7 minutes.

**Development of Lateral Flow Assay (LFA) Components of the LFA:** The LFA test strip used in the development of immuno chromatographic assay kit contained four key components; sample pad (for sample application), nitrocellulose membrane (contains test and control lines), conjugate pad and absorbent pad. To prepare whole card, all 4 pads are assembled in a side by side manner on self-adhesive card with a backing card support. After proper assembling, the card was cut into strips, then strips were housed inside a plastic cassette. The cassettes have openings on two windows at sample pad and nitrocellulose membrane where test and control lines are visible. Purified r3ABC protein, biotin BSA and protein G gold colloid were used as reagents in making lateral chromatographic assay. The nitrocellulose membrane was coated with r3ABC protein at test area and biotin-BSA was coated as control line by using Bio-jet apparatus (BioDotQuanti-200). A fiber mat containing 40 mm diameter protein G Gold Colloidal Nanoparticles (Genomix, USA) were used as detecting reagent. At last the test cassette was sealed in a moisture resistant pouch along with silica gel and dropper for sample application. The assay optimization was carried out by step-wise procedure with a reference panel of positive and negative samples. The assay execution was performed by placing test cassette on a clean surface and adding 5 ml of serum sample along with 50 ml of sample dilution buffer (1X PBS) to the sample pad. After application of sample, the sample will pass through the device along the conjugate pad, nitrocellulose membrane and at last the absorbent pad. The results were recorded within 20 min on the basis of appearance of colored line on test and control lines. The positive test results were analyzed by the presence of red or brown colored band in test and control lines, negative results by a colored band observed at only control line and invalid result by a clear band at test line and no clear line at control line (Fig. 2).

The specificity and sensitivity of FMDV lateral flow assay was evaluated by testing 33 reference control sera samples and 20 negative control samples. After testing 380 field samples (all the samples tested in duplicates) with LFA and ELISA and the LFA test was compared with the commercial FMDV ELISA assay and sensitivity and specificity of the assay was analyzed by employing the below formula.

\[
\text{Specificity} = \frac{TN}{(FP+TN)} \times 100 \\
\text{Sensitivity} = \frac{TP}{(TP+FN)} \times 100
\]

**Results**

A fragment of 1326 bp of FMDV 3ABC gene was cloned into the expression plasmid pET-28a (+) and transformed into E. coli BL21 cells for the production of r3ABC protein. The produced recombinant 3ABC protein having His-tag was purified using Ni-NTA column chromatography and during purification processes, the elutes were fractionated. The fractionated elutions resulted a
60 kDa distinct band on SDS PAGE analysis and Coomassie brilliant blue staining (Fig.1). Purified recombinant protein was used as antigen for the LFA and validated the assay with reference sera obtained by third-party. While the positive reaction was observed with FMD infected sera samples, the colored bands noticed at test and control lines appeared with negative sera bands at control line only. Among the 33 positive reference sera, 31 showed positive reaction with LFA and 33 with ELISA assay. Among the 20 negative controls, 19 samples showed negative results with both LFA and ELISA assay and 1 sample displayed positive with LFA. Also, 380 field samples were tested with LFA and the results were compared with the commercial ELISA results (Table 1). Among 180 vaccinated animal sera, 26 were positives with LFA and 29 with ELISA. Among the 200 non-vaccinated samples, 8 were positive with LFA and 8 with ELISA. The specificity of the LFA was 95.23% and the sensitivity was 94.28% (Table 2).

Discussion

In the current study, we have evaluated FMD r3ABC antibody detection test for rapid antibody detection in FMDV-infected animals. This FMD LFA test allows the rapid antibody detection in field and is useful for differentiating vaccinated from infected animals. FMD is a highly contagious viral disease of domestic ungulates and causes severe economic loss for livestock industry due to losses in milk production (13), and increased risk of abortion and cause of mortality among young animals. Presently, the FMDV infections are detected by virus capture in sandwich ELISA, virus isolation, neutralization assays, and PCR-based assays. These assays are reliable but need trained laboratory persons and also take more time to obtain test results (14). At present, FMD vaccination program is only the more effective medicament against the foot and mouth disease, but there is a problem in differentiating infected animals from vaccinated animals. Vaccination-based disease controlling operational program is effective in India with regular six-month vaccination programs and monitoring immuno-reactive antibody levels in the herds. Detection of infected animals from vaccinated animals is the important disease controlling measurement. NSP-based immunoassays (15) are the sensitive techniques to detect infected from vaccinated herd. For getting good results, researchers developed ELISA test.
to detect the antibodies to NSPs with Differentiating Infected from Vaccinated Animals (DIVA) strategies as per OIE recommended non-structural protein group, 3ABC polyprotein or individual proteins like 3A, 3B, 2C (OIE, 1995) (6). The similar profiling immunoassay using NSPs have been developed in India for FMDV. Previous studies (16) used individual antigens and developed an indirect ELISA to detect diseased animals from vaccinated animals. Eventually multiple antigens were used for FMD control. In South Africa, 3ABC polyprotein was used as an antigen in ELISA development for obtaining better sensitivity and specificity (17). DIVA test was developed for FMD using recombinant protein 3AB C for the surveillance of infection in vaccinated herd in India in the year 2009. Usage of DIVA test would be helpful for identification of disease-free areas in India where vaccination was performed. In India, the northern states are becoming disease-free with the regular vaccination programs and sero-monitoring. But, screening of animals with the ELISA assay for FMDV requires well-trained laboratory personnel, sophisticated labs and takes more time and high cost to perform the test. For a large number of populations, this type of test is not affordable. Therefore, it is important to have a pen-side test which can be performed at door step. The lateral flow immunoassay is a diagnostic device which incorporates immunoassay technique with rapid chromatographic principle and coating highly specific antibodies and antigens on nitrocellulose membrane. The lateral flow assay has been developed extensively to instigate immunological diagnosis of a vast number of diseases, including FMD (18). In the past few years, higher demand was noticed for pen-side tests with multiplex test lines which permit quick and simultaneous detection of different biological components present in the sample (19). Besides, lateral flow assay does not require specific expertise and special equipment and also no refrigeration for storage of the device (20). Hence, in the current study, LFA was evaluated with ELISA. The results illustrate that a pen-side, point-of-care LFA for the detection of FMDV antibodies to r3ABC in animals has been successfully developed. The test results

<table>
<thead>
<tr>
<th>S. No</th>
<th>Sample names</th>
<th>No. of Samples</th>
<th>LFA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>Vaccinated sera samples</td>
<td>180</td>
<td>26</td>
<td>154</td>
</tr>
<tr>
<td>2</td>
<td>Non-vaccinated serum samples</td>
<td>200</td>
<td>8</td>
<td>192</td>
</tr>
<tr>
<td>3</td>
<td>Reference positive controls</td>
<td>33</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Negative control sera</td>
<td>20</td>
<td>1</td>
<td>19</td>
</tr>
</tbody>
</table>

Table 2. Sensitivity and Specificity of FMD 3ABC Lateral Flow Assay

<table>
<thead>
<tr>
<th>No. of Specimen</th>
<th>LFA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>ELISA</td>
<td>66</td>
<td>70</td>
</tr>
</tbody>
</table>

Total samples: 433

Sensitivity: 66/(66+4)×100=94.3%
Specificity: 363/(4+363)=98.9%

Development of point-of-care lateral flow immuno-chromatographic assay
can be available within 15 minutes and has 94% sensitivity and 98% specificity. The newly developed LFA has high specificity and can be the most useful rapid immunodiagnostic tool to detect infected animals in vaccinated herds.

Conclusions
In the current study, lateral flow FMDV antibody detection assay has been developed and sensitivity comparable to the ELISA test was accomplished and the results could be produced with in 15 min. The LFA has the ability to give rapid results and more specificity to detect r3ABC of FMDV at pen-side. The newly developed lateral flow rapid antibody assay of FMDV NSPs was ideal for the point-of-care diagnosis, reliable, quick, easy and most preferable test to screen the vaccinated animals and can be used to control and prevent the FMDV disease eradication in endemic regions.

References


Method development and estimation of phenylenediamine in gastric contents, blood and urine

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Abstract:

A method for detecting and estimating Para-phenylenediamine (PPD) in the biological samples has been developed and validated using High-Performance Liquid Chromatography (HPLC) with UV/VIS detector. The method quantitatively measured the PPD in the biological samples. The mobile phase was composed of 10: 90 (Acetonitrile: Ammonium acetate 25 Mm v/v) of pH 4.5. The isocratic separation was carried out at C18 silica gel column at 1ml/minute of flow rate. The spectrophotometric detection was carried out at 240 nm. The process was validated, and the limit of detection for PPD was found to be 10μg. The accuracy and precision of the process were within limits. The linearity of the process found to be upto 1000 ppm. The extraction of PPD was carried out with chloroform and Sodium Hydroxide. The stock solution of PPD was freshly prepared due to oxidation potential. Aniline was used as an internal standard. The sample negative and sample positive were run to ensure the validity of the process. The estimation of the PPD was performed by drawing the calibration curve of different calibrators of PPD. The determination of PPD was carried out in gastric contents, Blood, and Urine. The extracted samples were prepared and run at HPLC for the detection. The pretreatment of the samples was not required, and the method proved to be accurate, precise, and specific.

Key words: Phenylenediamine; Pharmaceutical Science; Method development

Introduction

Para-Phenylenediamine (PPD), an organic compound and derivative of paranitroaniline compound has been traditionally used in dying and rubber industry, because of its oxidative chemical reaction PPD penetrates into hairs and produces colour to the hair. Accumulation of a large amount of the compound in the body may cause the following response such as skin, respiratory and ophthalmic reactions, moreover this synthetic compound will produce much life-threatening situation in many developing countries by producing intentional and unintentional intoxication (1).

It has been a long tradition to use such chemicals which are used to dye hairs and skin. The first synthetic hair dye was manufactured in

Phenylenediamine in gastric contents, blood and urine
Hair dyes consist of the colorless developer, coupler and hydrogen peroxide an oxidising agent. PPD has been used commercially for more than a decade. These belong to permanent oxidative hair dyes that belong to a vast group of hair colourants (2). For the most part, p-phenylene-diamine (PPD), paminophenol, toluone-2,5-diamine or different arylamines are utilised as developers. Separated from being utilised within hair formulations PPD and its derivative serve as beginning materials for amalgamating azo dyes, antioxidants, tattoo colours and pharmaceuticals. Furthermore, they have sought colour photography and as quickening agents to synthesise polymer fibres (3). The primary purpose of this research is to build up the method and estimation of p-phenylenediamine in a biological sample by using HPLC.

**Materials and methods**

This research project was performed at the Institute of Biochemistry and Biotechnology and WTO Laboratory, University of Veterinary and Animal Sciences, Lahore.

**Experimental design:** PPD in pure form is readily available in the local market. The PPD was orally administered to the rabbits, and biological samples (i.e. gastric fluid, heart, blood and urine) were collected by dissecting the test animal. The concentrations of samples were estimated by HPLC done by UV-Vis detector. The estimation is done for the concentration of paraphenylenediamine in the biological samples. Thirty rabbits (19 male and 11 female) weighing from 0.95 kg – 1.70kg were selected as biological samples for the test. 250 mg/kg is the acute toxic dose of PPD in rabbits. Blood samples were drawn after 1 hour of ingestion of PPD to rabbits. Other biological samples, such as gastric fluid and urine, were collected from the rabbits immediately after death. Biological samples from the test animals (blood, urine and gastric content) were collected and were stored at biological refrigerator (-4 °C).

**Chemicals and instrumentation:** PPD Standard (Standpharm Pakistan (Pvt.) Ltd.), Aniline as IS (Standpharm Pakistan (Pvt.) Ltd.), Ammonium Acetate (Standpharm Pakistan (Pvt.) Ltd), Acetonitrile HPLC/Spectro Grade (M.W 41.1, Assay 99.9%, MP Biomedicals, LLC, France, HPLC grade Water, Distilled water. High Performance Liquid Chromatograph is used. Shimadzu HPLC system fitted with autosampler (SIL-20A), reciprocating pump (LC-20AT), and solvent delivery system (LC-20A) with system controller (CBM-20A), detection was done by a UV-Vis detector at wavelength of 240 nm and Merck C18 column (15 cm x 3.9 mm x 5u) was used.

**Preparation of standard:** PPD 1000 mg was dissolved in 500 mL of distilled water to make 1000 ppm solution and make up the final volume 1000 mL in the volumetric flask. The stock solution was freshly prepared daily because of the oxidative degradation of the PPD. A dilution of 20-120ppm solutions were prepared to make the calibration curve. Aniline was used as Internal Standard. It is not present in biological samples. Further, it produced reproducible results. Aniline (IS) 100 mg was dissolved in 50 mL of distilled water to make 1000 ppm solution and made up the final volume 100 mL in the volumetric flask. To prepare 100 mL of 500 ppm solution, 50 mL of the stock IS solution was diluted with 30 mL of mobile phase in a volumetric flask and, in the end, the final volume made up to 100 mL with the mobile phase. The retention time of target analyte (PPD) and internal standard (Aniline) was determined by running 100 ppm solutions of each, separately, on HPLC.

**Preparation of positive control:** To prepare 100 mL of 0.2% w/v positive quality control, 200 mg of PPD standard was accurately weighed and dissolved in 50 mL 10 mL Acetonitrile solution and make the volume to 100 mL by Ammonium acetate buffer. The solution was refrigerated before use. This was a reference solution or test solution used for assessment of the performance of the procedure.

**Preparation of negative control:** Acetonitrile 10% solution and ammonium acetate 90% (25Mm)
buffer was used as a negative control sample. It was PPD free sample. This was a reference solution or test solution used for assessment of the performance of the procedure.

**Preparation of samples:**

**Gastric contents sample preparation:** The organic extraction procedure was employed for the preparation of the sample for the detection of PPD. 2ml of sample is added with 2ml chloroform and 100 microliter internal standard mixed for 15 minutes, then separate the organic layer. Repeat the same procedure for further purification and complete extraction without an internal standard for 3 times. The organic layer was dried by nitrogen purging. Mix the dried extract with the mobile phase. The solution was further processed for the estimation of PPD.

**Blood sample preparation:** The 2mL whole blood sample is added with 100 microliter of internal standard and was subjected to centrifugation. The plasma of the sample is added with chloroform, and the extraction proceeded. The extracted sample is dried under nitrogen and reconstituted with the mobile phase. This solution was further processed for the estimation of PPD.

**Urine sample preparation:** 2mL urine sample 2 mL, is added with 100 microliter of internal standard, 0.1 N NaOH and 2ml of chloroform. The solution was mixed for 15 minutes. The organic layer separated was added with chloroform; the procedure is repeated thrice. The extracted sample was then dried by nitrogen purging. The sample was reconstituted with the mobile phase. This solution was further processed for the estimation of PPD.

**Procedure:** The method for the detection of PPD in biological samples was optimised. Extraction was performed after the addition of Internal Standard. 10ml volume of all calibrator, Positive QC, Negative QC and all samples were pipette out in 15 mL centrifuge tubes. This produced the final strength of 100 ppm of IS. The extraction procedure was done by the addition of chloroform and 0.1N NaOH and mixed for 15 minutes. The chloroform layer was separated and purified by repeating the extraction for 3 times. The extracts were dried under nitrogen gas. The dried extracts were dissolved in 10 mL of mobile phase and ran on HPLC.

**Preparation of mobile phase:** Mobile phase was composed of Acetonitrile: Ammonium acetate solution (10:90). The Ammonium acetate solution was of 25mM strength having pH of 6.5. Scientific Working Group for forensic Toxicology (SWGTOX) guidelines for validation of quantitative analysis was followed. SWGTOX is the group of forensic toxicology experts from America and another world who set forensic toxicology laboratories standards in the world (4). The following validation parameters were evaluated:

**Linearity and calibration model:** The linearity/calibration samples were prepared using simulated gastric contents. The concentrations prepared were 0.04mg/g, 0.24mg/g, 0.48mg/g, 2.38mg/g, 4.75mg/g, and 23.7mg/g of zinc phosphide equivalent to 10μg/g, 50μg/g, 100μg/g, 500μg/g, 1000μg/g and 5000μg/g of PPD respectively. Each concentration level was evaluated in five different runs on, and concentration versus mean area curve for each concentration was drawn.

**Accuracy and precision:** Three levels were selected to establish accuracy and precision. These levels were prepared by fortifying the blank gastric contents at concentrations 50μg/g, 100μg/g and 1000μg/g. Different concentrations of PPD ranging from 0.5μg/g to 50μg/g were prepared by spiking the appropriate amount of PPD in simulated gastric contents and run on HPLC to find LOD. Various concentrations of PPD from 1μg/g to 50μg/g were prepared by spiking PPD into the simulated gastric contents and run on HPLC to find LOQ. Guidelines were taken from quality control analytical methods (5).

**Chromatographic conditions:** Shimadzu HPLC system fitted with autosampler (SIL-20A), reciprocating pump (LC-20AT) and Merck C18 column (15 cm x 3.9 mm x 5u) was used for
separation (6)(7). A Shimadzu HPLC solvent delivery system controller (LC-20A) with system controller (CBM-20A) was used. The 20μL sample was used in the injection system. Detection was done by UV-Vis detector at a wavelength of 240 nm. The instrument’s response to increasing concentrations of analyte was found to be linear over the range of 10μg/g to 1000μg/g. However, the linearity was broken above the 1000μg/g of PPD. The peak area for each concentration in five different runs is given in the table below.

**Results**

The linearity study results indicated that the method provides a linear response within the range of the most probable amount of PPD in the stomach contents (Figure 1). When unintended analyte appears in the blank sample after analysing the positive sample, it is called carryover. Carryover is evaluated during method validation for quantitative as well as qualitative analysis. It was found that no carryover was observed up to the concentration of 1000μg/g of PPD. However, little carryover peak was observed after the 5000μg/g of P7 PPD level but its integrated area was less than 10% of the smallest area of 10μg/g level.

Accuracy studies are usually conducted in concurrent with precision studies. At least three levels are selected at low, medium and high concentrations, and fortified blank samples of these levels are analysed for five days. Following formula was used to calculate accuracy at each concentration level.

\[
\text{Accuracy at Concentration} = \frac{\text{Mean of Measured Concentrations} - \text{Known Concentration}}{\text{Known Concentration}} \times 100
\]

The results of measured amounts of each concentration level are presented in the table below (Table 2). Accuracy and precision for each concentration level were calculated from the mean measured amount of the five days’ data.

![Validated Linearity Curve](Fig 1: Validated Linearity Curve)

**Table 1:** Data for Accuracy and Precision Studies

<table>
<thead>
<tr>
<th>Days</th>
<th>50 μg/g</th>
<th>100 μg/g</th>
<th>1000 μg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44.5</td>
<td>129</td>
<td>997.3</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>83.9</td>
<td>997.5</td>
</tr>
<tr>
<td>3</td>
<td>41.1</td>
<td>125.7</td>
<td>684.8</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>101.1</td>
<td>989.5</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>118.4</td>
<td>944.1</td>
</tr>
<tr>
<td>Mean</td>
<td>47.2</td>
<td>111.6</td>
<td>922.64</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>8.5</td>
<td>18.87</td>
<td>135</td>
</tr>
</tbody>
</table>

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Limit of detection and limit of quantification:

Limit of detection (LOD) is the minimum amount of the analyte that can be detected by analytical procedure (8). In the study the LOD was found to be 10 μg/g. The peak produced at the retention time of PPD i.e.; 2.317 minutes was reproducible at the concentration of 10 μg/g. below this concentration, the response was not reproducible. Limit of Quantification is the minimum concentration that can be quantitated, and method can produce multiple symmetrical results. 1000 μg/g was the minimum concentration at which the multiple runs produced symmetrical peaks, and the results were within ±20% of accuracy and %CV of less than 20%. So the LOQ found to be was 1000 μg/g.

Retention time measurement:

100 ppm of PPD solution was run, and it gave a retention time of 2.320 minutes. Further, 100 ppm solution of Aniline (internal standard) produced a peak at 15.660 minutes showing its retention time. Negative QC gave a retention time of 5.287 min and did not show PPD peak. 0.2% w/v PPD solution was run, and it gave retention time of 8.665 min, and Aniline (internal standard) produced a peak at 5.337 min showing its retention time. Sample AHZ B1 was Blood. Its chromatographic profile shows that AUC of the PPD is 46797465 and that of (IS) is 16445487. Sample AHZ B2 was Blood. Its

### Table 2: Summary of validation results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Desired limit</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration Model</td>
<td>50-1000 μg/g (linear model desired)</td>
<td>50-1000 μg/g Linear model</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Must not exceed ±20%</td>
<td>92.3 to 111.6 %</td>
</tr>
<tr>
<td>Precision</td>
<td>%CV must not exceed 20%</td>
<td>14.6 to 18 %</td>
</tr>
<tr>
<td>Limit of Detection (LOD)</td>
<td>Must be 3 μg/g or lower</td>
<td>10 μg/g</td>
</tr>
<tr>
<td>Limit of Quantitation (LOQ)</td>
<td>Must be 10 μg/g or lower</td>
<td>1000 μg/g</td>
</tr>
<tr>
<td>Carryover</td>
<td>Carryover after highest calibrator does not exceed 10% of signal of lowest calibrator</td>
<td>No carryover</td>
</tr>
</tbody>
</table>

Phenylenediamine in gastric contents, blood and urine
chromatographic profile shows that AUC of the PPD is 46906162 and that of (IS) is 15113134. Sample AHZ B3 was Blood. Its chromatographic profile shows that AUC of the PPD is 30134559 and that of (IS) is 14127024. Sample AHZ B4 was Blood. Its chromatographic profile shows that AUC of the PPD is 30014839 and that of (IS) is 14221388. Sample AHZ B5 was Blood. Its chromatographic profile shows that AUC of the PPD is 30134862 and that of (IS) is 14189546. Sample AHZ B6 was Blood. Its chromatographic profile shows that AUC of the PPD is 48825438 and that of (IS) is 1419546. Sample AHZ B7 was Blood. Its chromatographic profile shows that AUC of the PPD is 44797777 and that of (IS) is 16156445. Sample AHZ B8 was Blood. Its chromatographic profile shows that AUC of the PPD is 46747497 and that of (IS) is 14245444. Sample AHZ B9 was Blood. Its chromatographic profile shows that AUC of the PPD is 46798777 and that of (IS) is 16245444. Sample AHZ B10 was Blood. Its chromatographic profile shows that AUC of the PPD is 46474497 and that of (IS) is 16479844. Sample AHZ G11 was gastric content. Its chromatographic profile shows that AUC of the PPD is 41796895 and that of (IS) is 16638495. Sample AHZ G12 was gastric content. Its chromatographic profile shows that AUC of the PPD is 43977751 and that of (IS) is 16916781. Sample AHZ G14 was gastric content. Its chromatographic profile shows that AUC of the PPD is 49663343 and that of (IS) is 15690167. Sample AHZ G15 was gastric content. Its chromatographic profile shows that AUC of the PPD is 45457874 and that of (IS) is 16979794. Sample AHZ G16 was gastric content. Its chromatographic profile shows that AUC of the PPD is 46549498 and that of (IS) is 16444654. Sample AHZ U21 was urine. Its chromatographic profile shows that AUC of the PPD is 985347 and that of (IS) is 1410361. Sample AHZ U22 was urine. Its chromatographic profile shows that AUC of the PPD is 1040181 and that of (IS) is 1200902. Sample AHZ U23 was urine. Its chromatographic profile shows that AUC of the PPD is 94776 and that of (IS) is 1485271. Sample AHZ U24 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889. Sample AHZ U25 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889. Sample AHZ U26 was urine. Its chromatographic profile shows that AUC of the PPD is 821681 and that of (IS) is 1300566. Sample AHZ U27 was urine. Its chromatographic profile shows that AUC of the PPD is 94776 and that of (IS) is 1485271. Sample AHZ U28 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889. Sample AHZ U29 was urine. Its chromatographic profile shows that AUC of the PPD is 2546 and that of (IS) is 1534889. Sample AHZ U30 was urine. Its chromatographic profile shows that AUC of the PPD is 1149796 and that of (IS) is 1692019.

**Discussion**

Mainly, para-phenylenediamine (PPD), paminophenol, toluone-2,5-diamine or other aryamines are used as developers. Apart from being used in hair formulations PPD and its derivatives serve as starting materials to synthesize azo dyes, antioxidants, tattoo colours and pharmaceuticals. In addition, they are applied for colour photography and as accelerators for the synthesis of polymer fibres (3).
Table 3: Standard Deviation From The Mean In Blood Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>AUC</th>
<th>Regression constant (a)</th>
<th>Regression constant (b)</th>
<th>Concentration of PPD (ppm)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>46809018</td>
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<tr>
<td>B2</td>
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<td>2.00E+06</td>
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<td>B3</td>
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</tr>
<tr>
<td>B4</td>
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<tr>
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<td>2.00E+06</td>
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<tr>
<td>B8</td>
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<td>289210</td>
<td>2.00E+06</td>
<td>154.7232</td>
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</tr>
<tr>
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<td>2.00E+06</td>
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</tr>
<tr>
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<td>46909492</td>
<td>289210</td>
<td>2.00E+06</td>
<td>155.2833</td>
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</table>

Table 4: Standard Deviation From The Mean In Urine Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>AUC</th>
<th>Regression constant (a)</th>
<th>Regression constant (b)</th>
<th>Concentration of PPD (ppm)</th>
<th>SD</th>
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</thead>
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<td>2.00E+06</td>
<td>105.3692</td>
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<tr>
<td>U2</td>
<td>20040976</td>
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<td>2.00E+06</td>
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<tr>
<td>U3</td>
<td>32786089</td>
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<td>2.00E+06</td>
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<tr>
<td>U4</td>
<td>28726878</td>
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<td>U5</td>
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<td>2.00E+06</td>
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<tr>
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<td>2.00E+06</td>
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Phenylenediamine in gastric contents, blood and urine
### Table 5: Standard Deviation From The Mean In Gastric Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>AUC</th>
<th>Regression constant (a)</th>
<th>Regression constant (b)</th>
<th>Concentration of PPD (ppm)</th>
<th>SD</th>
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<tbody>
<tr>
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<tr>
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<tr>
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<td>2.00E+06</td>
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<td>2.00E+06</td>
<td>145.1463</td>
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</tr>
<tr>
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<td>289210</td>
<td>2.00E+06</td>
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<tr>
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<td>2.00E+06</td>
<td>150.2641</td>
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<tr>
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**Fig 2: Chromatogram of Retention time of PPD**

**Fig 3: Chromatogram of Retention time of Aniline**

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According to Axel Meyer at al 2009 (9) developed a method for the quantitative detection of PPD using HPLC. The mobile phase was composed of acetonitrile; ammonium acetate buffer(5:95, 25mM v/v). The limit of quantification of PPD was determined at 0.5 μM for PPD. In the present study, a method for the detection and estimation of Para phenylenediamine in the biological samples has been developed and validated using HPLC UV/VIS. The method could quantitatively measure the PPD in the biological samples. The mobile phase was 10: 90 (ACN: ammonium acetate 25 Mm v/v) of pH 4.5. The isocratic separation was carried out at C18 silica gel column at 1ml/minute of flow rate. The spectrophotometric detection was carried out at

Phenylenediamine in gastric contents, blood and urine
**Fig 6:** Chromatographic profile of samples (A)AHZ B1 (B) AHZ B2 (C) AHZ B3 (D) AHZ B4 (E) AHZ B5 (F) AHZ B6 (G) AHZ B7 (H) AHZ B8 (I) AHZ B9

**Fig 7:** Chromatographic profile of samples (J)AHZ B10 (K) AHZ B11 (L) AHZ B12 (M) AHZ B13 (N) AHZ B14 (O) AHZ B15 (P) AHZ B16 (Q) AHZ B17 (R) AHZ B18 (S) AHZ B19 (T) AHZ B20 (U) AHZ B21

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The process was validated, and the limit of detection for PPD was found to be 10 μg. The accuracy and precision of the process was within the limits. The linearity of the process found to be up to 1000 ppm. The interday and intraday precision and accuracy were within limits. The extraction of PPD was carried out with chloroform and NaOH. The Stock solution of PPD was freshly prepared due to oxidation potential. Aniline was used as an internal standard. The sample negative and sample positive were run to ensure the validity of the process. The estimation of the PPD was performed by drawing the calibration curve of different calibrators of PPD. The determination of PPD was carried out in Gastric contents, Blood, and Urine. The extracted samples were prepared and run at HPLC for the detection. The pretreatment of the samples was not required, and the method proved to be accurate, precise, and specific.

References

Phenylenediamine in gastric contents, blood and urine
Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. Journal of Analytical Toxicology, 37(7), 452-474.


Abstract
Herbal medicine has proven to be one of the most well-known fields of traditional medicine worldwide. Extracts from different traditional medicinal plants have been screened to discover the source of therapeutic effects and nature has been a source of medicinal agent for thousands of years. Antioxidants protect the body from the harmful damage produced by free radical induced oxidative stress. The present study is the assessment of the total flavonoid contents and antioxidant potential of medicinal plants. Different parts of the plant were used namely the stem bark of Schima wallichi (DC) Korth., Millettia pachycarpa Benth., the leaves of Eleagnus caudata Schlecht, Dysoxylum gobara Buch.-Ham and the fruit of Castanopsis indica (Roxb.)A.DC. The chloroform and ethanol extracts of all these plants showed free radical scavenging activity in a concentration dependent manner. S. wallichi showed the maximum scavenging activity followed by E. caudata, M. pachycarpa, C. indica and D. gobara accordingly. The chloroform and ethanol extracts both showed an increase in the flavonoid content in a concentration dependent manner. The amount of total flavonoids was higher in the ethanol extracts than that of chloroform extracts. The ethanol extracts also has greater flavonoid content and possess higher antioxidant activity when compared to the chloroform extracts. Our study concluded that S. wallichi showed highest ABTS scavenging activity among all the five plants. The antioxidant activity was not directly proportional to the total flavonoid contents of a plant species. The amount of total flavonoids was found to be lower in S. wallichi, which shows that other secondary compound like alkaloids, phenols, etc may have contributed to this effect.

Key words: ABTS; Total flavonoids; Medicinal plants.

Introduction
In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous (1). For thousands of years nature has been the major source of medicine (2). Bioactive plants and their extracts have the potential to provide novel products for prevention and treatment of diseases. Due to its effectiveness in treating various diseases, minor side effects and cheaper cost, medicinal plants are highly popular among developing countries (3). Medicinal plants are indispensable to the global economy. Many secondary metabolites are commercially sold and are crucial to many pharmaceutical companies (4).

In India, the traditional knowledge of medicinal plants used by indigenous people are well documented and has been passed on the next generation for many years (5). Phytochemicals are natural chemical compounds found in plants. Plants produce these chemicals as a mode of defense mechanism for their protection, but these phytochemicals also provide protection...
for humans from diseases according to recent studies (6). Flavonoids are ubiquitous in nature and occurs as glycones, glycosides and methylated derivatives in vascular plants. Flavonoids are polyphenolic compounds (7). Due to their extensive biological and pharmacological properties, flavonoids have been widely studied. Flavonoids are potent antioxidants that can protect human beings from free radicals and reactive oxygen species. Their antioxidant capacity and scavenging activities depends upon their molecular structure, mainly on the position of hydroxyl groups in its chemical structure (8). Flavonoids contain many substances that protect biological systems from the toxic effects of oxidative processes (9). Free radicals have harmful effects on human beings and is related to toxicity and causing diseases like diabetes, chronic renal failure, cancer, mellitus, atherosclerosis, immune dysfunction and aging (10). Food sources like fruits and vegetables contain many free radical scavenging antioxidants (11). Free radicals have a damaging effect on cells and cause various degenerative disorders when they are produced excessively (12).

Antioxidants can intervene the production of free radicals during the main steps of the free radical mediated oxidative processes, viz., initiation, propagation and termination (13). Most living organisms have an effective defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS) (14). Due to the certain health benefits, less toxicity, cheap price and accessibility, antioxidants from plant sources are getting popular (15). Therefore, this study targets to investigate antioxidant potential of different medicinal plants including Schima wallichii, Millettia pachycarpa, Eleagnus caudata, Castanopsis indica and Dysoxylum gobara in vitro by evaluating ABTS scavenging activity.

Medicinal uses:
Schima wallichii (DC) Korth: The leaves and the stem bark are traditionally used for its medicinal properties. The bark is used as an antiseptic for wounds. It is also used as a vermicide, and treating gonorrhea (16), decoction of bark is effective against head lice and reduces fever. The bark juice of Chilauni is used in animals as a liver fluke disinfesting agent (17). People of Western Mizoram use the fruit juice of Chilauni for treating snakebite (18). Schima wallichii has anti-cancer activities, and have the ability to induce apoptotic mechanisms (19).

**Elaeagnus caudata Schlecht**: The fruit is taken as a health tonic (20). The extract of the fruit or stem bark is mixed with *Piper longum* and is taken for 2-3 weeks on a daily basis to cure jaundice and other liver troubles (21). The root decoction is taken to expel the retained placenta, ease labor and as a treatment after child birth. The leaf infusion is used for strengthening the function of uterus after child birth (22).

**Millettia pachycarpa Benth**: *M. pachycarpa* is used in Chinese traditional medicine for the preparation of ’Jixueteng’ that induce the growth of red blood cells (23). The compounds isolated from *M. pachycarpa* has been reported to be cytotoxic and induce apoptosis in HeLa cells (24) and also show cytotoxic effect in Brine shrimp assay (25) with anti-inflammatory activity (26). In India and China, it is used traditionally in treating cancer and infertility. It is also used as a pesticide and as a blood tonic (27). The bark paste is also used in treating diseases like skin infections and itches (28).

**Castanopsis indica (Roxb.) A. DC**: *C. indica* is traditionally used for treating stomach disorders, chest pain, skin diseases, headache and diarrhea (29). The leaf decoction is used to treat stomach disorder and skin diseases (30). The seeds are consumed raw in Nepal (31) and Mizoram (32). The resin is given to treat diarrhea and the leaf paste is applied for headache. The bark paste is also applied on the chest to control chest pain (33).

**Dysoxylum gobara Buch.-Ham**: The leaf and bud decoction is used to treat diarrhea and dysentery (34-36). The tender leaves and flowers are cooked
and eaten as a vegetable. The decoction of leaves is used as a remedy for food poisoning, diarrhea and dysentery (37).

**Materials and Methods**

**Chemicals:** Ascorbic acid, trolox and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), aluminium chloride, chloroform, ethanol, potassium acetate, quercetin, potassium persulfate.

**Collection of Plant Materials:** The healthy stem bark of *Milletia pachycarpa* Benth., *Schima wallichi* (DC) Korth., the leaves of *Eleagnus caudata* Schlecht., *Dysoxylum gobara* Buch.-Ham. and the fruit of *Castanopsis indica* (Roxb.) A.DC. were collected during the dry season from different parts of Mizoram. The plant specimens were identified by Prof. Lalramnghinglova, Department of HAMP, Mizoram University, Aizawl. The herbarium specimens are deposited at the Department of Zoology, Mizoram University. The stem bark, leaves or fruits were examined visually for infection, washed thoroughly with clean water and allowed to shade dry at RT in the dark, clean and hygienic conditions. The dried plant material was powdered using an electrical grinder at room temperature and sequentially extracted with chloroform and ethanol using a Soxhlet apparatus. The liquid extracts were filtered and dried using rotary vacuum evaporator and stored at -70°C until further use.

**Total flavonoid content:** The total flavonoid content was determined by AlCl₃ method (38). The ethanol and chloroform extracts of different concentrations were mixed with 1.5 ml of methanol, 0.1 ml of 10% AlCl₃, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. Then it was incubated at RT for 30min. The absorbance was measured at 415 nm with double beam UV spectrophotometer. The calibration curve was prepared by preparing Quercetin solution at different concentrations.

**Antioxidant capacity using ABTS scavenging assay:** The ABTS cation scavenging activity was determined for the different extracts using a minor modification of Re R et al (39). 37.5 mg of potassium persulfate was taken and dissolved in 1 ml of distilled water. 44 ml was taken from this solution and dissolved in 2.5 ml of distilled water with 9.7 mg of ABTS. The absorbance was measured at 734 nm after the solution was kept in dark condition at RT for 16 hours. The results have been represented as ascorbic acid equivalent. The scavenging activity was calculated using the following formula:

\[
\text{Inhibition (\% )} = \frac{\text{(A control OD-Atest OD)}}{(\text{A control OD})} \times 100
\]

**Results and Discussion**

Phytochemicals found in plants possesses significant benefits to human health such as ascorbic acid, carotenoids and phenolic compounds (40). Phytochemical compounds that naturally possess anticarcinogenic (41-42) and other beneficial properties are referred to as chemo-preventers and this protective action is due to their antioxidant activity and their capacity to scavenge free radicals (43).

The total flavonoid contents of chloroform extracts of *Milletia pachycarpa*, *Schima wallichi*, *Eleagnus caudata*, *Castanopsis indica* and *Dysoxylum gobara* showed a concentration dependent rise up to 2500 μg/ml (Figure.1). The highest total flavonoid was present in *E. caudata* (130.36±2.15 mg/g) followed by *D. gobara* (94.62±1.58 mg/g), *M. pachycarpa* (51.27±2.14 mg/g), *C. indica* (48.61±3.40 mg/g) and *S. wallichi* (26.82±1.25 mg/g). The least total flavonoid content was detected in *S. wallichi*.

The presence of total flavonoid contents in ethanol extracts of all five plants also showed an increase in flavonoid content with an increase in concentration manner. The highest flavonoid content were also found at 2500 μg/ml for all the extracts with the highest value present in *Dysoxylum gobara* (75.73±0.98 mg/g), followed by *E. caudata* (53.43±3.27 mg/g), *C. indica* (38.04±23 mg/g), *S. wallichi* (32.06±2.29 mg/g). The lowest content was found in *M. pachycarpa* (19.43±0.71 mg/g). The order of the flavonoid content was determined for the different extracts using a minor modification of Re R et al (39). 37.5 mg of potassium persulfate was taken and dissolved in 1 ml of distilled water. 44 ml was taken from this solution and dissolved in 2.5 ml of distilled water with 9.7 mg of ABTS. The absorbance was measured at 734 nm after the solution was kept in dark condition at RT for 16 hours. The results have been represented as ascorbic acid equivalent. The scavenging activity was calculated using the following formula:

\[
\text{Inhibition (\% )} = \frac{\text{(A control OD-Atest OD)}}{(\text{A control OD})} \times 100
\]
content observed was as follows: ECC (130.36±2.15) > DGC (94.62±1.58) > DGE (75.73±0.98) > ECE (53.43±3.27) > MPC (51.27±2.14) > CIC (48.61±3.40) > CIE (32.06±2.29) > SWE (26.82±1.25) > MPE (19.43±0.71) at the concentration 2500 μg/ml. The present study revealed high content of both phenols and flavonoids (Figure 1), both flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties (44).

The chloroform extracts of the five plants showed a concentration dependent rise up to 5000 μg/ml in the ABTS scavenging activity. The maximum ABTS inhibition for chloroform extract was found in *S. wallichi* (78.45±0.95 μg/ml) followed by *M. pachycarpa* (63.44±2.58 μg/ml), *E. caudata* (63.28±2.44 μg/ml) and *D. gobara* (22.93±0.53 μg/ml). The ethanol extract of all the plants also showed the highest ABTS scavenging at 5000 μg/ml. The ethyl alcohol extract of *M. pachycarpa* showed the highest activity (89.02±1.79 μg/ml), followed by, *S. wallichi* (88.84±2.46 μg/ml), *E. caudata* (88.60±0.94 μg/ml), and *C. indica* (79.90±1.65 μg/ml) and *D. gobara* (78.67±2.21 μg/ml).

The order of scavenging activity was observed as follows: MPE (89.02±1.79%) > SWE (88.84±2.46%) > ECE (88.60±0.94%) > CIE (79.90±1.65%) > DGE (78.67±2.21%) > SWC (78.45±0.95%) > CIC (63.44±2.58%) > MPC (63.28±2.44%) > ECC (63.28±2.44%) > DGC (22.93±0.53%) at the concentration 5000 μg/ml.

The analysis of ABTS scavenging in respect of TROLOX equivalent was similar as maximum scavenging activity was observed at concentration of 5000 ig/ml for chloroform extracts and ethyl alcohol extracts of all the five plants.

Correlation analysis of total flavonoid content and scavenging activity of certain medicinal plants of Mizoram, extracted with chloroform and ethyl alcohol shows that an increase in the flavonoid content causes an increase in the scavenging activity. The chloroform extracts of *M. pachycarpa*, *S. wallichi*, *E. caudata*, *C. indica* and *D. gobara* did inhibit the generation of ABTS free radicals in concentration dependent manner and this activity was highest at the concentration of 5000 μg/ml with EC50 values of 2140 μg/ml, 475.9 μg/ml, 74.18 μg/ml, 2295 μg/ml respectively, however *D. gobara* did not show insignificant effective concentration compared to the others, the maximum effect was observed for 5000 ig/ml with 22.93±0.53% of scavenging activity.

**Fig. 1:** The total flavonoid content of various medicinal plant of Mizoram extract with chloroform (a) and ethyl alcohol (b) as Quercetin equivalent. The values represented as Mean+ SEM, n=3.
The assessment of the free radical scavenging activity of medicinal plants
remained almost similar up to a concentration of 5000 μg/ml. The comparison between activity and content showed positive correlation and the order of positive correlation was as follows: CIE (r = 0.99) > ECC (r = 0.975) > CIC (r = 0.951) > DGC (r = 0.950) > MPC (r = 0.93) > SWC (r = 0.88) > DGC (r = 0.85) > ECE (r = 0.82) > MPE (r = 0.71) > SWE (r = 0.55).

The free radical scavenging activity of plants extract against ABTS cations revealed significant reduction with less concentration (Figure 2). The active metabolites present in the plants are responsible for the antioxidant activity and the natural phenol and flavonoid contents are present in fruit, leaves, flower and the seeds of plants (45). The TROLOX equivalent activity showed the plant extract possesses high efficacy of scavenging properties (Figure 3). The correlation analysis of both concentration and activity revealed that the activity increased with increase in concentration (Table 1). The natural antioxidants maybe useful as they may have fewer side effects or no side effects, because of their biologic origin. *E. caudata* scavenged the ABTS free radicals in a concentration dependent manner indicating its antioxidant potential. The other species of *Eleagnus* including *E. angustifolia* have been reported to scavenge ABTS radical (46). *S. wallichi* inhibit the generation of ABTS free radicals in a concentration dependent manner and this activity was highest for this plant (Figure 2). The present study correspond with the previous reported by (47, 17, 48). The resulting antioxidant effect observed was almost akin to the present study. The chloroform and ethanol extracts of *M. pachycarpa* did inhibit the generation of DPPH free radicals in concentration dependent manner. The ethyl alcohol extract of *M. pachycarpa* showed the highest activity.

*M. pachycarpa* has been known to scavenge free radicals (49-50) and the results are also in accordance with the findings in this study. *C. indica* scavenged the ABTS free radicals in a concentration dependent manner indicating...
its antioxidant potential. The *C. indica* has been reported to scavenge superoxide, hydroxyl and ferric free radicals earlier and this activity was attributed to the presence of phenolic compounds (51).

The other species of *Castanopsis* including *Castanopsis cuspidate* have been reported to have antioxidant property (52).

The antioxidant activity of *D. gobara* was lesser than the other plants and possesses the least antioxidant activity among all the five plants. *D. gobara* has been reported to possess antioxidant activity in DPPH assays earlier (50) and the results are also in accordance with the findings in the present study. The other species of *Dysoxylum* namely *Dysoxylum cauliflorum* have been reported to possess antioxidant activity in both the DPPH scavenging and FRAP assay.

**Conclusion**

The present study of medicinal plants including *M. pachycarpa, S. wallichi, E. caudata, C. indica* and *D. gobara* revealed high content of bioactive compound and potential antioxidant efficiency, which might be useful resources for future ethno-medicine. However, the mechanism of action of secondary metabolites required further investigation in future.

**Acknowledgement**

The authors express their gratitude to CSIR-UGC, New Delhi, India for providing financial assistance to Alex Zohmachhuana, Dept. of Botany.

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The assessment of the free radical scavenging activity of medicinal plants
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46. Okmen, G. and Turkcan, O. A Study on Antimicrobial, Antioxidant and Antimutagenic Activities of Elaeagnus Angustifolia L. Leaves Afr J Tradit


Assessment of eco-genotoxic effects of pesticide mixtures on freshwater fish, Catla catla

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*Corresponding author: kjreddy32@gmail.com

Abstract
Eco-genotoxicity was evaluated in fresh water fish, Catla catla using two commercially available pesticide mixtures, viz., Deltamethrin 1% + Triazophos 35% EC and Profenofos 40% EC + Cypermethrin 4% EC. Acute toxicity studies were conducted primarily to determine LC₅₀ of each pesticide mixture. The fish were exposed to each pesticide mixture for 21 days at a dose value of 1/20th and 1/10th of LC₅₀. The gills were isolated from the fish at the end of the exposure period, and processed for comet assay. Nucleoids were scored visually, observed significant increase of damage and categorized into various degrees of damages. Based on the results obtained from this study it is concluded that the pesticide mixtures evaluated could be potentially genotoxic to fresh water fish, Catla catla.

Key words: Eco-genotoxicity, acute toxicity, comet assay, DNA damage.

Introduction
The current scenario to assess risk, particularly the regulatory limits for the use of pesticides mixtures are based on selective toxicological tests of the individual pesticides in the mixture and not on the basis of combined toxic effects of the pesticides in the mixtures. The aim of the most of the toxicity studies being conducted usually with individual pesticides or mixtures of pesticides on aquatic organisms are to understand mortality, behavioral changes and sometimes hematological and biochemical changes. The reports on genotoxicity induced by the pesticides or in combination on fish are very limited. The reason to choose fish for assessment of eco genotoxicity is because of sensitive to pesticide residues and other toxic pollutants. Fish accumulates pollutants dissolved in water and respond to toxicants in a similar way to higher vertebrates (1, 2). Carcinogenic activities such as formation of tumors in different tissues of fish exposed to insecticide may be the cause of xenobiotic used. The eco-genotoxic activity of certain pesticides can reduce the fitness of wild fish production, and also pose high risk to human health via food chain (3). Endocrine disruptors being used in insecticides can change the expression of vital genes that are responsible for reproductive dysfunction or immunosuppression (4).

The pesticides that are proved to be non genotoxic using existing battery of tests may or may not be non genotoxic for ecologically important organisms. The analysis of DNA changes in aquatic organisms will be a useful tool to evaluate contaminants existed in the aquatic environments with genotoxic compounds (5,6). Chromosomal aberrations, micronuclei formation, sister chromatid exchange and comet assay are the frequently using biomarkers to assess genotoxic effects of pesticides (7). Single cell gel electrophoresis (SCGE) or comet assay are the inexpensive techniques have been used for the past few years for measuring and analyzing DNA single and double-strand breaks, DNA cross-
linking and delayed repair-site detection in eukaryotic individual cells (8, 9). Based on the availability of limited literature on combination of pesticide mixtures in the present study ecogenotoxic effects of two pesticides mixtures were evaluated in the freshwater fish, *Catla catla*.

**Materials and methods**

**Acute toxicity:** Two pesticides mixtures viz., Deltamethrin 1% + Triazophos 35% EC (D+T(EC)) and Profenofos 40% EC + Cypermethrin 4% EC (P+C(EC)) were procured commercially. Fresh water fish (*Catla catla*) (6 – 7 cm in length and approximately 6g in weight) were procured from a commercial supplier. Fish were transported to the laboratory in aerated water and quarantined for 12 days. Prior to initiation of the experiment fish were, acclimatized for 7 days and feed was withdrawn for 24 h before conduct of the study. Fish were fed with commercially available fish feed during the acclimatization and quarantine period. The acute toxicity of pesticide mixtures in fish was determined based on OECD guideline 203 (10) and Guidance document on toxicology for registration of pesticides in India, 2017 (11).

The temperature of the test room and the test medium was maintained between 21 - 25°C and a photoperiod of 12 h light and 12 h darkness was maintained using a timer. Blended water (A mixture of well water and reverse osmosis water in the ratio of 1:1) was used as the exposure medium. Ten fish each were exposed to 5 different test concentrations (Table 1) of each pesticides mixture and blended water as a control for 96 h. The concentrations of pesticides were prepared separately and transferred to glass aquaria containing 20 L of blended water. The exposure media were renewed at the end of every 24 h with the respective concentrations or blended water. An initial study was performed to assess the acute toxicity of the pesticides mixtures with various concentrations of D+T (EC), viz., 0.1, 0.5, 1, 5, 10mg/L and P+ C (EC) viz., 0.05, 0.1, 0.5, 1.0, 5.0 mg/L. Fish were observed for mortality and morbidity at 3 h and 6 h at the start of exposure and thereafter at the end of 24, 48, 72 and 96 h.

Physico-chemical parameters such as pH, dissolved oxygen, temperature were analyzed daily in the exposure media (12). LC$_{50}$ values for different concentrations of pesticides mixture were calculated by AAT Bioquest® calculator (13).

**Sub-lethal toxicity:** Ten fish each were exposed to sub-lethal concentrations (1/20th and 1/10th of LC$_{50}$ values) of each pesticide’s mixtures for 21 days. The 1/20th and 1/10th of LC$_{50}$ values were 0.05 and 0.10 mg/L, respectively for D+T (EC), 0.02 and 0.05 mg/L, respectively for P + C (EC). A concurrent control was also maintained. The concentrations of the pesticides mixtures were prepared as given above. Exposure medium was renewed daily. The fish were fed with commercially available fish feed once in two days. The fish were observed for morbidity and mortality if any, daily.

**Genotoxicity (Comet assay:** At the end of 21 days sub-lethal toxicity study, the exposed fish gills were isolated and minced using a mincing solution comprised of (HBSS, 20 mM EDTA, DMSO and Sterile water) with a micropestle and allowed to settle. The supernatant containing single cells extracted from the gills tissue was used for comet assay (9, 14). For the basal layer, 1% normal melting agarose in phosphate buffered saline (PBS) was prepared. To this about 25 μl of the cell suspension from each sample was mixed with 75 μl of low melting agarose (0.5% in PBS) covered with cover glass and allowed to solidify. After removal of the cover glass, the slides were immersed in 50 ml of cold lysing solution and maintained in dark condition at 4°C for 1 h. Slides were then transferred to a tank containing electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH >13), for 20 min to make the DNA unwinding. After electrophoresis, the slides were washed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 15 min and then stained with 75 μl of ethidium bromide (2 μg/mL) and screened for comets using a fluorescence microscope at 400X magnification. The cells were scored by their tail intensities and the scores were categorized as 0 (undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive) based on (15). The total amount

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of DNA strand breakage was expressed in total arbitrary units (AUT) defined as:

$$AUT = N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4,$$

where $N_i$ is the number of nuclei scored in each category (16).

**Statistics:** Statistical evaluation was performed using SPSS 16.0 version.

**Results and discussion**

**Acute toxicity:** It's a continuous process that aquatic organisms are being exposed to various pollutants in the environment. A group of organisms such as plants, animals, fish or wildlife under controlled conditions when exposed to toxic pollutants can be easily evaluated the toxicity. Pesticides can produce adverse effects in a biological system, seriously damaging its structure and function of living system finally leads to death of organism. Those adverse responses may be defined in terms of a measurement as acute toxicity. Pesticides are entering into aquatic ecosystem by agriculture runoff from land, impairing the quality of the water and making it unfavorable for aquatic life (17).

**Treatment with Pesticide mixture D+T (EC):** No mortality was observed in control and fish treated with D+T (EC) at a concentration of 0.1 and 0.5 mg/L, whereas 30% mortality was

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>No of Fish tested</th>
<th>Mortality at</th>
<th>Mortality (%) upto 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3h</td>
<td>6h</td>
<td>24h</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 1.** Mortality in *Catla catla* exposed to various concentrations of D+T (EC) at 3, 6, 24, 48, 72 and 96 hours (h).

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
<th>No of Fish tested</th>
<th>Mortality at</th>
<th>Mortality (%) upto 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3h</td>
<td>6h</td>
<td>24h</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2.** Mortality in *Catla catla* exposed to various concentrations of P+C (EC) at 3, 6, 24, 48, 72 and 96 hours (h).

Eco-genotoxic effects of pesticide mixtures on freshwater fish, *Catla catla*
observed in fish exposed to the concentration of 1 mg/L after 96 h. Fish exposed to the concentration of D+T (EC) at 5 mg/L exhibited 100% mortality at the end of 48 h, while fish exposed to 10 mg/L exhibited 100% mortality at the end of 24 hrs (Table 1). Fish exposed to the concentrations of 1, 5 and 10 mg/L exhibited clinical signs such as pigmentation, loss of equilibrium, rapid opercular movement and lateral lying at the bottom of the aquaria. Acute toxicity study conducted by (18) revealed that Triazophos exposed to fish individually at lower concentrations indicates no mortality but when exposed to higher concentrations showed death after 2-3 hours of exposure period.

**Treatment with Pesticides mixture P+C (EC):** Control and fish treated with P+C (EC) at a concentration of 0.05 and 0.1 mg/L showed no mortality until 96 h, whereas 40% mortality was observed in fish exposed to the concentration of 0.5 mg/L at 72 h. Fish exposed to the concentration of 1 mg/L exhibited 80% mortality at the end of 48 hrs, respectively (Table 2). Similarly, 100% mortality was observed when fish treated with 5 mg/L at 48 hrs. Fish exposed to the concentrations of 0.5, 1 and 5 mg/L exhibited toxicity signs such as pigmentation and loss of equilibrium. A dose dependent increase and time dependent decrease were observed in *Catla catla* when exposed to Profenofos individually and the mortality rate at the exposure time increased from 24 to 96 hours (18).

**Determination of LC<sub>50</sub>:** Based on the above mortality data, the LC<sub>50</sub> were determined by AAT Bioquest® calculator (13) and results were incorporated in Table 3.

Table 4. Analysis of DNA damage as measured by comet assay in gills tissue of *Catla catla*.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Proportion of damaged nuclei</th>
<th>%DNA Damage (1+2+3+4)</th>
<th>DNA Damage score (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.00 23.00 0.90 0.10 0.00</td>
<td>24 25.1</td>
<td></td>
</tr>
<tr>
<td>0.05mg/L</td>
<td>46 29 14 7 2</td>
<td>49 83</td>
<td></td>
</tr>
<tr>
<td>0.10mg/L</td>
<td>29 30 31 14 6</td>
<td>81 158</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>45.67 27.67 17.00 7.67 2.33</td>
<td>55.00 94.00</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>2.52 1.53 3.00 3.06 0.58</td>
<td>7.21 14.18</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>25.67 27.00 29.33 13.67 6.33</td>
<td>76.33 152.00</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>3.06 2.65 2.08 3.51 0.58</td>
<td>6.43 13.08</td>
<td></td>
</tr>
</tbody>
</table>

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Table 3. LC<sub>50</sub> of pesticides mixtures for *Catla catla*

<table>
<thead>
<tr>
<th>Pesticides mixtures</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+T (EC)</td>
<td>1.09</td>
</tr>
<tr>
<td>P+C (EC)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Physico-chemical parameters: The physico-chemical parameters in the exposure media determined during the experiments were, pH - 7.2 – 8.0; temperature - 21.2 – 23.5°C; dissolved oxygen - 75 – 108%; hardness - 201 – 209 mg/L and conductivity - 741 – 783 μs/cm, and were within the acceptable range (12).

Table 5. Analysis of DNA damage as measured by comet assay in gills tissue of *Catla catla*.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Proportion of damaged nuclei</th>
<th>%DNA Damage (1+2+3+4)</th>
<th>DNA Damage score (AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>78.00</td>
<td>19.00</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>18</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>25</td>
<td>0.8</td>
</tr>
<tr>
<td>Average</td>
<td>79.33</td>
<td>20.67</td>
<td>0.63</td>
</tr>
<tr>
<td>SD</td>
<td>1.53</td>
<td>3.79</td>
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<tr>
<td>0.02</td>
<td>39</td>
<td>23</td>
<td>15</td>
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<td></td>
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<tr>
<td></td>
<td>42</td>
<td>23</td>
<td>10</td>
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<tr>
<td>Average</td>
<td>40.67</td>
<td>24.33</td>
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<tr>
<td>SD</td>
<td>1.53</td>
<td>2.31</td>
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<tr>
<td>SD</td>
<td>3.00</td>
<td>0.58</td>
<td>0.58</td>
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</table>

Sub-lethal toxicity: Mortality and any toxicity signs were not observed in control and in fish exposed to the sub-lethal concentrations of the pesticides mixtures for 21 days.

Comet assay (genotoxicity in fish): DNA damage if any by the pesticide mixtures was analyzed and observed that the pesticide mixtures, D+T (EC) (Table 4) and P+C (EC) (Table 5) induced strand breaks, when exposed to the concentrations of 0.05, 0.10 mg/L and 0.02, 0.05mg/L, respectively. In the present study, the pesticides mixtures exhibited a significant increase (p < 0.05) in the % DNA damage compared to the control. The DNA damage observed could possibly be initiated from DNA single or double strand breaks or through the formation of DNA adducts and/or DNA cross links, which might have resulted due to the interaction of DNA with the pesticides mixtures (19, 20).

Eco-genotoxic effects of pesticide mixtures on freshwater fish, *Catla catla*
Studies of this nature are useful to monitor the ecosystem health and, consequently, for the well-being of all the organisms exposed to it, including man (21).

Conclusion
On analysis it was concluded that at lower concentrations, pesticides may not be toxic to aquatic organisms directly/immediately but may alter the genomic function of the organisms as revealed in the present study where the sub-lethal concentration of the pesticides mixtures did not show any signs of toxicity, but caused genotoxicity. However, from the comet assay one can point out only the general damages to the DNA and cannot specify the region, affected, which may impair the growth, reproduction and population dynamics of the organisms in the long term exposure which in turn gradually leads to the extinction of these species, further molecular studies are essential to understand about the mode of action of these chemicals on genome of the beneficial organisms, on its DNA repair mechanisms and the genome area, affected.

References
alkaline comet assay on fish and earthworm tissues. Environmental Molecular Mutagen, 41: 85-91.


Eco-genotoxic effects of pesticide mixtures on freshwater fish, *Catla catla*
Abstract
A new analytical “reverse phase high performance liquid Chromatography (RP-HPLC) assay method has been developed for estimation of Levofloxacin in injection phase. The separation was achieved by using column Inertsil ODS-3V (250 x 4.6mm, 5 μm) mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v). The flow rate was 1.0mL.min⁻¹. Levofloxacin was detected using UV detector at the wavelength of 293 nm. The retention time of Levofloxacin was noted to be 11.20 min respectively. The method was evaluated as per ICH guidelines. The proposed method was found to be advantageous than the existing methods towards accuracy, reproducibility, and consistent.

Key words: RP-HPLC, Levofloxacin, Forced degradation and Validation.

Introduction
Levofloxacin hemihydrate is highly water and organic solvents like glacial acetic acid and chloroform, sparingly soluble in methanol, slightly soluble in ethanol, and practically insoluble in ether. Levofloxacin hemihydrate is odourless drug. Methods for quantitative analysis of Levofloxacin by HPLC [1–7], by UV [8–10] spectroscopy in single as well as in combination, are available in the literature. The method was developed and validated as per ICH [11–13] and USP [14] guideline.

Materials and Methods
Chemicals and reagents: Analytical-grade Ammonium acetate, Citric acid monohydrate and Hydrochloric acid was from Merck chemicals Mumbai, India. Methanol, Acetonitrile and water, both HPLC-grades, were from Merck chemicals. Mumbai, India. Millex syringe filters (0.45 im) were from Millex-HN, Millipore Mumbai, India.
Instrumentation: Agilent HPLC model: 1260 with DAD, Bandelin ultrasonic bath, pH Meter (Thermo Orion Model), Analytical Balance (Metller Toledo Model) were used.

Preparation of 0.05 M solution of citric acid monohydrate: Accurately weighed and dissolved 10.5 g of citric acid monohydrate in 1000 ml of water and sonicated to dissolved and mixed well.

Preparation of 1.0 M Ammonium acetate solution: Accurately weighed and dissolved 7.71 g of ammonium acetate in 100 ml of water and sonicated to dissolved and mixed well.

Preparation of buffer solution: Mixed accurately 840 ml of a 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate solution.

Mobile phase: Mixed buffer solution and acetonitrile in the ratio of 85:15 v/v, filtered and degassed.

Blank preparation: Use Milli-Q water.

Standard preparation: Accurately weighed 50.0 mg of Levofloxacin working standard or reference standard was transferred into a 50 ml volumetric flask. Added 7.5 ml of 0.1 M Hydrochloric acid solution stirred well and diluted to the volume with 0.1 M Hydrochloric acid solution. Transferred 5.0 ml of resulting solution into a 25 ml volumetric flask and diluted to volume with water and mixed well. The solution was diluted to volume with diluent and mixed well. (Concentration of Levofloxacin is about 0.2 mg/ml).

Placebo solution: Transferred 10 ml of the placebo solution, added 7.5 mL of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well.

Sample preparation: Transferred 10 ml of the sample solution into a 50 mL volumetric flask, added 7.5 ml of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well.

Further transferred 5.0 ml of the resulting solution into a 25 mL volumetric flask and diluted to volume with water and mixed well.

Chromatographic conditions: Chromatographic analysis was performed on 250x4.6mm, 5μm column. The mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v). The flow rate was 1.0mL/min, column oven temperature 25°C, the injection volume was 10IL, and detection was performed at 293 nm using a photodiode array detector (PDA).

Results and discussion
Method development: The Spectral data of compound Levofloxacin showed that maximum UV absorbance (emax) at 293 nm. To develop a suitable and robust LC method for the determination of Levofloxacin, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Inertsil ODS-3V, 250x4.6mm, 5μm with the following different mobile phase compositions of 0.05 M solution of citric acid monohydrate buffer and acetonitrile in the ratio of 85:15 v/v. It was observed that when Levofloxacin was injected, higher retention time, Peak Tailing are not satisfactory. For next trial the mobile phase consisted of 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of 85:15 v/v was employed at the flow rate of 1.0 mL/min. UV detection as performed at 293nm. The retention time of Levofloxacin is 11.20 minutes and the peak shape was good. The chromatogram of Levofloxacin standard using the proposed method is shown in Figure: 1.2 system suitability results of the method are presented in Table: 1.2.

Method validation: The developed RP-HPLC method extensively validated for assay of Levofloxacin using the following parameters.
Specificity & System suitability:

Preparation of blank solution: Used Milli-Q water as a blank solution.

Preparation of Placebo solution: Transferred 10 ml of the placebo solution into a 50 mL volumetric flask, added 7.5 mL of 0.1 M solution of hydrochloric acid, and diluted to volume with mobile phase and mixed well. Further transferred 5.0 ml of the resulting solution into a 25 mL volumetric flask and diluted to volume with water and mixed well.

Blank and Placebo interference: A study to establish the interference of blank and placebo were conducted. Diluent and placebo was injected into the chromatograph in the defined above chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of blank solution Figure:1.3 showed no peak at the retention time of Levofloxacin peak. This indicates that the diluent solution used in sample preparation do not interfere in estimation of Levofloxacin in Levofloxacin injection. Similarly chromatogram of placebo solution Figure: 1.4 showed no peaks at the retention time of Levofloxacin peak. This indicates that the placebo used in sample preparation do not interfere in estimation of Levofloxacin in Levofloxacin injection.

Table 1.1: Specificity results for Levofloxacin

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Placebo solution</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Standard solution</td>
<td>11.24</td>
</tr>
<tr>
<td>4</td>
<td>Sample solution</td>
<td>11.22</td>
</tr>
</tbody>
</table>

Table 1.2: System suitability parameters for Levofloxacin

<table>
<thead>
<tr>
<th>No.of injections</th>
<th>Tailing factor</th>
<th>Tailing plates</th>
<th>Theoretical Area of Levofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inj-1</td>
<td>1.4</td>
<td>7622</td>
<td>1088768955</td>
</tr>
<tr>
<td>Inj-2</td>
<td>1.4</td>
<td>7580</td>
<td>1086835821</td>
</tr>
<tr>
<td>Inj-3</td>
<td>1.4</td>
<td>7590</td>
<td>1088318629</td>
</tr>
<tr>
<td>Inj-4</td>
<td>1.4</td>
<td>7527</td>
<td>1089977609</td>
</tr>
<tr>
<td>Inj-5</td>
<td>1.4</td>
<td>7598</td>
<td>1086265129</td>
</tr>
<tr>
<td>Average %RSD</td>
<td></td>
<td></td>
<td>1088033229</td>
</tr>
</tbody>
</table>

Development and validation of stability indicating RP-HPLC method.
Forced Degradation study: The study involves assessing the effect of acid (0.1N HCl, 2 hrs at 60°C temperature), base (0.1N NaOH, 2 hrs at 60°C temperature), hydrogen peroxide (3%, 2 hrs at 60°C temperature), Thermal (105°C for 48 hours) and UV light (7 days) on Levofloxacin injection samples. The chromatograms obtained from various stress conditions are shown in Figure: 1.5. The percent assay, percent degradation and peak purity of Levofloxacin and retention time of degradants produced in all stress conditions are determined and summarized in Table: 1.3. Levofloxacin was found to be more stable in applied acid, base, thermal and photolytic stress conditions. Levofloxacin was sensitive to adopted stress condition like oxidation. The results proved that the developed assay method has good selectivity and specificity, and is suitable for assay of Levofloxacin in the presence of stress degradation products.

Method precision: The precision of test method was evaluated by doing assay for six samples of Levofloxacin injection as per test method. The content in mg and % label claim for Levofloxacin for each of the test preparation was calculated. The average content of the six preparations and % RSD for the six observations were calculated. The chromatogram was shown in Figure: 1.6 and data were shown in Table: 1.4.
Development and validation of stability indicating RP-HPLC method
Table: 1.4. Method precision data for Levofloxacin

<table>
<thead>
<tr>
<th>No. of injections</th>
<th>Levofloxacin % assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>106.2</td>
</tr>
<tr>
<td>2</td>
<td>105.7</td>
</tr>
<tr>
<td>3</td>
<td>105.3</td>
</tr>
<tr>
<td>4</td>
<td>105.1</td>
</tr>
<tr>
<td>5</td>
<td>104.5</td>
</tr>
<tr>
<td>6</td>
<td>105.4</td>
</tr>
<tr>
<td>Average</td>
<td>105.4</td>
</tr>
<tr>
<td>SD</td>
<td>0.57</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Intermediate Precision: The intermediate precision of test method was demonstrated by carrying out method precision study in six samples, representing a single batch by two different analysts on two different days, different column, different HPLC system and by different analyst. These samples were prepared as per the test method. The % assay was calculated for each of these samples. The precision of the method was evaluated by computing the % Relative standard deviation of % assay of Levofloxacin.
Table 1.5 Intermediate precision data for Levofloxacin

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Area of Levofloxacin</th>
<th>Assay of Levofloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>186778849</td>
<td>104.4</td>
</tr>
<tr>
<td>2.</td>
<td>187067790</td>
<td>104.6</td>
</tr>
<tr>
<td>3.</td>
<td>187523508</td>
<td>104.9</td>
</tr>
<tr>
<td>4.</td>
<td>187333783</td>
<td>104.8</td>
</tr>
<tr>
<td>5.</td>
<td>187033298</td>
<td>104.6</td>
</tr>
<tr>
<td>6.</td>
<td>186616560</td>
<td>104.4</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>104.6</td>
</tr>
<tr>
<td>%RSD</td>
<td></td>
<td>0.19</td>
</tr>
</tbody>
</table>

- Overall and individual % of Assay are complies as per test method specification.
- The relative standard deviation of six assay preparations is **0.19**.
- The overall relative standard deviation of six assay preparations of precision study and six assay preparations of intermediate precision study is **0.54**.

**Linearity of detector response:** The standard curve was obtained in the concentration range of 100.07-300.21\(\mu\)g/ml for Levofloxacin. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient \([r]\) of standard curve were calculated and given in Figure 1.7 to demonstrate the linearity of the proposed method. From the data obtained which given in Table 1.6 the method was found to be linear within the proposed range.

Table 1.6 Linearity studies for Levofloxacin by proposed method

<table>
<thead>
<tr>
<th>% Concentration</th>
<th>Levofloxacin</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>100.0713</td>
<td>590667579</td>
</tr>
<tr>
<td>75</td>
<td>152.1083</td>
<td>900530185</td>
</tr>
<tr>
<td>100</td>
<td>200.1425</td>
<td>1157439141</td>
</tr>
<tr>
<td>125</td>
<td>252.1796</td>
<td>1461276205</td>
</tr>
<tr>
<td>150</td>
<td>300.2138</td>
<td>1737499643</td>
</tr>
<tr>
<td>%Y-intercept</td>
<td>1.99</td>
<td></td>
</tr>
</tbody>
</table>

**Accuracy:** The accuracy of the test method was demonstrated by preparing recovery samples of Levofloxacin at 50%, 75%, 100%, 125% and 150% of the target concentration level. The recovery samples were prepared in triplicate for each concentration level except 50% and 150% (50% and 150% are six preparations). The above samples were chromatographed and the percentage recovery of each sample was calculated for the amount added. Evaluated the precision of the recovery at each level by computing the Relative Standard Deviation of six preparations for 50% and 150% level recovery samples results. The percentage recoveries with found in the range of 99.0 to 99.9 for Levofloxacin. The chromatogram was shown in Figure 1.8 to 2.2 the data obtained which given in Table 1.7 the method was found to be accurate.

![Fig. 1.7. Calibration curve for Levofloxacin](image1)

![Fig. 1.8. Accuracy (Spike level 50%) chromatogram](image2)

Development and validation of stability indicating RP-HPLC method
Table: 1.7 Recovery studies for Levofloxacin by proposed method

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Recovery</th>
<th>Mean % Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% sample-1</td>
<td>100.9</td>
<td>101.0</td>
<td>0.27</td>
</tr>
<tr>
<td>50% sample-2</td>
<td>100.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% sample-3</td>
<td>101.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% sample-4</td>
<td>101.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% sample-5</td>
<td>101.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% sample-6</td>
<td>100.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75% sample-1</td>
<td>101.0</td>
<td>101.3</td>
<td>0.3</td>
</tr>
<tr>
<td>75% sample-2</td>
<td>101.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75% sample-3</td>
<td>101.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% sample-1</td>
<td>101.8</td>
<td>101.8</td>
<td>0.25</td>
</tr>
<tr>
<td>100% sample-2</td>
<td>102.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% sample-3</td>
<td>101.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125% sample-1</td>
<td>100.9</td>
<td>100.7</td>
<td>0.25</td>
</tr>
<tr>
<td>125% sample-2</td>
<td>100.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125% sample-3</td>
<td>100.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150% sample-1</td>
<td>101.5</td>
<td>101.4</td>
<td>0.28</td>
</tr>
<tr>
<td>150% sample-2</td>
<td>101.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150% sample-3</td>
<td>101.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150% sample-4</td>
<td>101.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150% sample-5</td>
<td>101.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150% sample-6</td>
<td>101.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Robustness studies: To validate the method robustness the chromatographic performance at changed conditions was evaluated compared to nominal conditions of the method. Standard solution was injected at each of the following changed conditions:

Table: 1.8. Robustness studies Results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Theoretical plates</th>
<th>Tailing factor</th>
<th>%RSD of peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow variation ±10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+10%</td>
<td>8599</td>
<td>1.4</td>
<td>0.07</td>
</tr>
<tr>
<td>-10%</td>
<td>9820</td>
<td>1.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Temperature variation ±5°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+5°C</td>
<td>8467</td>
<td>1.3</td>
<td>0.16</td>
</tr>
<tr>
<td>-5°C</td>
<td>8126</td>
<td>1.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Mobile phase Variation ±10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+10%</td>
<td>7622</td>
<td>1.4</td>
<td>0.10</td>
</tr>
<tr>
<td>-10%</td>
<td>8064</td>
<td>1.4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

- Method is robust for changes like column oven temperature, flow rate and organic phase of mobile phase.

Prasad Babu and Ramachandran
Solution stability of analytical solutions:
Levofloxacin standard and sample solutions were kept for about 48 hrs at room temperature in transparent bottles in auto sampler and in refrigerator 2-8°C. The response of these was compared with respect Initial standard solution and sample solution.

Table: 1.9. Results for solution stability of standard at room temperature

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>similarity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>-</td>
</tr>
<tr>
<td>24hrs</td>
<td>1</td>
</tr>
<tr>
<td>48hrs</td>
<td>1</td>
</tr>
</tbody>
</table>

Table: 2.0 Results for solution stability of standard in Refrigerator

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>similarity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>-</td>
</tr>
<tr>
<td>24hrs</td>
<td>1</td>
</tr>
<tr>
<td>48hrs</td>
<td>1</td>
</tr>
</tbody>
</table>

Table: 2.1 Results for solution stability of standard at room temperature

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>% Assay</th>
<th>% of Assay difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>106.2</td>
<td>NA</td>
</tr>
<tr>
<td>24hrs</td>
<td>107.52</td>
<td>1.32</td>
</tr>
<tr>
<td>48hrs</td>
<td>107.07</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Table: 2.2 Results for solution stability of standard in Refrigerator

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>% Assay</th>
<th>% of Assay difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>106.2</td>
<td>NA</td>
</tr>
<tr>
<td>24hrs</td>
<td>107.85</td>
<td>1.65</td>
</tr>
<tr>
<td>48hrs</td>
<td>107.77</td>
<td>1.57</td>
</tr>
</tbody>
</table>

- Standard and sample solutions are stable for 48 hours when stored at room temperature (RT) and 2-8°C in refrigerator.

Development and validation of stability indicating rp-hplc method
Conclusion

An RP-HPLC method for estimation of Levofloxacin was developed and validated as per ICH guidelines. A simple, accurate and reproducible reverse phase HPLC method was developed for the estimation of Levofloxacin in bulk drugs and formulations. The optimized method consists of mobile phase 0.05 M solution of citric acid monohydrate and 10 ml of 1.0 M ammonium acetate buffer and acetonitrile in the ratio of (85:15 v/v) with Inertsil ODS-3V(250 x 4.6mm, 5μm) column. The retention time of Levofloxacin was found to be 11.20 minutes. The developed method was validated as per ICH Q2A (R1) guidelines. The proposed HPLC method was linear over the range of 100.07-300.21ppm, the correlation coefficient was found to be 0.9999. The percentage recoveries (accuracy) with found in the range of 99.0 to 99.9 for Levofloxacin. Relative standard deviation (%RSD) for method precision and intermediate precision was found to be 0.54 and 0.19. Solution stability of the Standard and sample solutions are stable for 48 hours when stored at room temperature (RT) and 2-8°C in refrigerator. Our developed method was considered as fast, simple and reliable analytical method for determination of Levofloxacin in pharmaceutical preparation using RP-HPLC. As there is no interference of blank and placebo at the retention time of Levofloxacin, it is very fast with good reproducibility and response. Validation of this method was accomplished and getting results to meet all the requirements. The method is simple, reproducible, with a good accuracy and Linearity. It allows reliably the analysis of Levofloxacin in its different pharmaceutical dosage forms.

Acknowledgment

The authors are grateful to Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur. Andhra Pradesh, India, for providing facilities to carry this research work.

Conflict of interests: The authors claim that there is no conflict of interest.
Development and validation of stability indicating RP-HPLC method
Formulation and evaluation of amoxicillin trihydrate oral lozenges for treating upper respiratory tract infections

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1 Chebrolu Hanumahia Institute of Pharmaceutical Sciences, Chandramoulipuram, Chowdavaram, Guntur, A.P., India.
*Corresponding author: svidyadhara@gmail.com

Abstract
In the present investigation an attempt has been made to develop Amoxicillin Trihydrate oral lozenges in treatment of upper respiratory tract infection. The lozenges were formulated by soft lozenges method employing Amoxicillin Trihydrate alone and in combination with natural antiseptic ingredients. The lozenges were prepared employing PEG 4000 as matrix base, Stevia (natural sweetener), Acacia (polymer), MCC (disintegrate) other excipients. The prepared medicated lozenges were characterized for weight uniformity, hardness, Drug content, and dissolution by standard pharmacopeia methods. The results of the evaluation tests obtained were within the limits. Formulations were tested for drug Excipient interactions by FTIR spectral analysis. The results revealed that there were no major interactions between the drug and polymers used for the preparation of lozenges. Antimicrobial activity studies were performed for different lozenges formulations. AMF2 formulation showed greater Zone of inhibition. This may be due to synergistic antimicrobial effect of Amoxicillin Trihydrate, Tulsi and Ginger. Accelerated stability studies were conducted as per ICH guidelines and found that there wasn’t any substantial change in the prepared formulations.

Key words: Amoxicillin Trihydrate, Ginger, Tulsi, PEG 4000, MCC, Stevia.

Introduction
Oral dosage forms have advantages over other dosage forms. They are economical and safe to the patient. They are appropriate for any patient, whatever the age is. Oral dosage forms have disadvantages as well. They are not the first choice of drugs if the patient suffers chronic vomiting. They are not good choice in case of uncooperative patients as children and infants. They are not suitable in emergency and for unconscious patients (1). In the 19th century, physicians discovered morphine and heroin, which suppress coughing at its source—the brain. Popular formulations of that era included Smith Brothers Cough Drops, first advertised in 1852, and Luden’s, created in 1879. Concern over the risk of opioid dependence led to the development of alternative medications (2). Lozenges historically have been used for the relief of minor sore throat pain and irritation and have been used extensively to deliver topical anesthetics and antibacterials. Today they are used for delivering the drugs for analgesics, anesthetics, antimicrobials, antiseptics, antiulssives, aromatics, astringents, corticosteroids, decongestants, and demulcents and other classes and combinations of drugs. Both chewing gum and lozenges may be considered as alternatives to current dosage forms. They are easy to handle, the dose has been apportioned, and the excipients have a demulcent effect on a sore throat since the ingredients are released slowly and spread uniformly over the affected mucosal membrane (3). Lozenges are placed in oral cavity. Since the sublingual lozenges may be impractical due to their size, buccal lozenges are formulated and have been extensively used and are intended to
be placed between the cheek and the gums. Though the lozenge dissolution time is about 30 minutes, it also depends on the patient, as patient controls the rate of dissolution and absorption by sucking on lozenge until it dissolves. Depending on the type of lozenge, they may be prepared by molding or by compression (4). Amoxicillin is a broad-spectrum, pharmacologically active beta-lactam antibiotic effective against Gram-positive and Gram-negative bacteria. It is a widely used antibiotic in human and veterinary medicine for the treatment and prevention of respiratory, gastrointestinal, urinary and skin infections due to its pharmacological and pharmacokinetic properties. Based on the above physicochemical and biopharmaceutical properties, amoxicillin trihydrate was selected as a drug candidate (5).

An herb is a plant or part of a plant valued for its medicinal, aromatic or savourly qualities. Nature produces several food items for every season. Their use in that particular season proves to be highly beneficial for the mankind which is packed with enormous medicinal advantages. Herbal drugs play a major role in systems of health in India; almost 70% of modern medicines in India is derived from natural products. In last few years there is an increment occur in the use of herbal medicines. The herbs used in herbal candy are selected on the basis of their role in the treatment of altitude health problems with lesser side effects, also the selection based on their availability and their preferences. The herbal products are much better than the allopathic medicines. Herbal products have lesser side effects and more therapeutic effects (6). Of all the herbs used within Ayurveda, tulsi (*Ocimum sanctum* Linn) is preeminent, and scientific research is now confirming its beneficial effects. There is mounting evidence that tulsi can address physical, chemical, metabolic and psychological stress through a unique combination of pharmacological actions. Tulsi has been found to protect organs and tissues against chemical stress from industrial pollutants and heavy metals, and physical stress from prolonged physical exertion, ischemia, physical restraint and exposure to cold and excessive noise. Tulsi's broad-spectrum antimicrobial activity, which includes activity against a range of human and animal pathogens, suggests it can be used as a hand sanitizer, mouthwash and water purifier as well as in animal rearing, wound healing, the preservation of food stuffs and herbal raw materials and traveler's health (7). On the other hand, ginger (*Zingiber officinale*) which is a member of the Zingiberaceaee (ginger) family, occurs in horizontal, laterally flattened irregularly branching piece; 3-16 cm long, 2-4 cm wide, up to 3 cm thick, sometimes split longitudinally, pale yellowish buff or light brown externally striated, somewhat fibrous, branches known as fingers arise obliquely from the rhizome, are flatish, ovibate, short, about 1-3 cm long, fracture, short and starchy with projecting fibers (8).

**Materials and Methods**

Amoxicillin Trihydrate (AMT) was obtained as gift sample from Apotex pharma Ltd, Bangalore; Tulsi and Ginger Powder were obtained from Patanjali Super Market, Guntur, Andhra Pradesh; Poly ethylene Glycol 4000, Hydrochloric acid, Acacia and MCC were obtained from S.D Fine Chem, Ltd, Mumbai; Silica gel and Citric acid monohydrate were obtained from High-Pure fine Chem, Chennai; Stevia Natural Sweetener was obtained from Procarvit Food Products (India) Private Ltd, Coimbatore.

**Estimation of amoxicillin trihydrate:** Standard solution of pure drug containing 100 mg of amoxicillin trihydrate /100 ml was prepared using 0.1 N HCl. The working standards were obtained by dilution of the stock solution in corresponding 0.1N HCl. The standard curve for amoxicillin trihydrate was prepared in concentration range of 5-25 μg/ml at the selected wave length of 227 nm. Their absorptivity values were to determine the linearity. Solutions were scanned and beer lamberts law limit was obeyed in concentration range of 0, 5, 10, 15, 20, 25 μg/ml using 0.1N HCl as blank.

**Solubility Studies (9):** Saturated solubility studies of AMT were performed in different
dissolution media. 100mg of AMT was weighed and transferred into different conical flasks containing 10ml of different dissolution media i.e., Water, 0.1N HCl, 6.8pH, 7.2pH Phosphate buffer and were closed appropriately. All the conical flasks were placed in a REMI incubator shaker at 50 rpm, 37°C ± 1°C for 24 hrs. The conical flasks were removed from the incubator shaker and samples were filtered using whattman filter paper. The clear solution obtained by filtration and was suitably diluted with appropriate dissolution media and the absorbance values were noted at 227 nm by using corresponding dissolution media as blank solutions. The absorbance values were noted. The solubility of amoxicillin in different media like 0.1N HCl (1.2 pH), pH 6.8 phosphate buffer, pH 4.6 acetate buffer and in distilled water (Table 1).

Preparation of soft lozenges: The quantity of each ingredient needed for compounding the preparation was calculated for 20 lozenges and the required material for two extra lozenges were calculated and weighed. Soft lozenges were prepared by melting and mold technique (2). The PEG (Grade of 4000) was placed into a small beaker (50 ml) and heated without stirring. The remaining powders were mixed in the geometric dilution technique by using mortar and pestle. The powder mixture was passed through a 40 mesh sieve onto a glassine sheet. Once the PEG was melted, the heat was reduced and a stir bar was added with lowest spin rate. The powders were sprinkled onto the melted PEG ensuring each addition is wetted before adding additional powder. Once the powders were added to the PEG, the beaker was removed from the hotplate and colour, flavor were added and allowed to cool until it is “just cool to the back of the hand.” The lozenge mold(s) were placed on an electronic balance and the weight of the mold(s) was tarred out. The lozenge material was poured into each mold cavity to the calculated desired weight per lozenge using the digital balance (Table 2 and Fig. 1).

Evaluation of Lozenges: The prepared formulations were evaluated for drug content uniformity, hardness, thickness and diameter, weight variation, friability and in vitro dissolution by pharmaceutical standard methods (Table 3).

In vitro Dissolution Studies for Formulated Lozenges: Dissolution studies were performed on lozenge formulations in a calibrated 8 station dissolution test apparatus (LABINDIA DS8000) equipped with paddles (USP apparatus II method) employing 900 ml of 0.1 N HCl as dissolution medium. The paddles were operated at 50 rpm and temperature was maintained at 37°C ± 1°C throughout the experiment. The samples were withdrawn at 5, 10, 15, 20, 30 and 45 minutes and replaced with equal volume of same dissolution medium to maintain the constant volume throughout the experiment. Samples were withdrawn at various time intervals and suitably diluted with same dissolution medium and the amount of the drug dissolved was estimated by ELICO double beam U.V spectrophotometer at 227 nm. The dissolution studies on each

Figure 1: Formulated Lozenges

Formulation and evaluation of amoxicillin trihydrate oral lozenges
formulation were conducted in triplicate. From the dissolution profiles various parameters like T<sub>50</sub>, and DE<sub>30%</sub> were calculated (Table 4 and Fig 2).

**Antimicrobial activity:** Antimicrobial activity was tested by Cup Plate Method depends on the diffusion of an antibiotic from a vertical cavity, through the solidified agar layer in a Petri plate. The microbial growth inhibited by the compound will despite as circular zone around the cavity. The nutrient agar is melted, cooled suitably, poured into Petri dish. Spread 0.2 ml of known concentration of inoculums on the surface of the solidified agar. Cavities are made by using a sterile borer. The lozenges formulation was dissolved in 6.8 pH phosphate buffer solution and poured into the cups of agar plate and then incubated for 18 hrs and the zone of inhibition (Fig. 3).

**Fourier Transform Infrared Spectroscopy:** Infrared spectra of drug and optimized lozenge formulations were recorded by KBr pellet method using Fourier Transform Infrared Spectrophotometer. Pure drug and optimized lozenge formulations were subjected for FTIR analysis using FTIR spectrophotometer to study any interactions between drug and excipients and spectra’s (Fig. 4).

**Accelerated stability studies:** The formulations which showed good *in-vitro* performance were subjected to accelerated stability studies. These studies were carried out by investigating the effect of temperature on the physical properties of lozenges and chemical stability of lozenges containing drugs. The lozenge formulations such as AMF<sub>3</sub> and AMF<sub>4</sub> were subjected to accelerated stability studies. The above said formulations were kept in petridishes after preparation and stored in thermostatic oven at a temperature and relative humidity of 25 ± 2°C, 60 ± 5% RH for 6 months

![Figure 2](Image) Drug Release Profiles of AMT Lozenges

![Figure 3](Image) Zone of Inhibitions observed for Anti Microbial Activity.

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and 40 ± 2°C, 75 ± 5% RH for 3 months. Then the samples of each type of formulations were evaluated for the earlier mentioned physical parameters. Further these lozenges were subjected to drug release studies as stated earlier (Fig. 5). The formulations subjected to Accelerated Stability studies were also characterized by FTIR the results revealed that there was no major interaction between the drug and excipients.

**Results and discussion**

The calibration curve for the estimation of Amoxicillin Trihydrate in 0.1 N HCl was found to be linear and obeyed Beer’s law in the concentration range of 5 - 25 μg/ml. Saturated solubility studies were conducted for Amoxicillin Trihydrate using different dissolution media. Amoxicillin Trihydrate showed maximum solubility in 0.1 N HCl medium. Preformulation studies were

![FTIR Spectroscopy](image)

*Figure 4: FTIR Spectroscopy (a) pure drug (b) AMF3 (c) AMF4*

![Drug Release Profiles](image)

*Figure 5: Drug Release Profiles of AMT Lozenge Formulation (AMF₃) Before and After Storage at Different Conditions*
performed on the drug and excipients used in the formulations and were found to be compatible. No drug and excipient reactions were observed.

The drug content of prepared Lozenges was found to be in the range of 246.8 – 249.8±0.3/mg. The melt and mold technique was found to be suitable for molding the soft lozenge formulations. All the batches of lozenges were compressed under identical conditions to minimize processing variables. The soft lozenges were prepared by using PEG 4000 in different concentrations i.e., 80 and 85%. All the prepared lozenge formulations were further evaluated for physical parameters. All the lozenge formulations were found to be stable and meeting I.P specified limits for weight uniformity and drug content. The hardness of all the lozenge formulations were in the range of 1-2 kg/cm². Weight uniformity of all the lozenge formulations were in the range of 125.14-142.25 ±3mg. Drug content estimated for all the lozenge formulations were highly uniform with less than 2.5% variation. The in-vitro dissolution studies for all the lozenge formulations were found to release the drug at a faster rate than compared to pure drug. It was found that the lozenge formulations AMF₃ and AMF₄ with 2.5 % and 2.6 % of silica gel and acacia as suspending agent showed the slow drug release when compared to other formulations. The rate of drug release of lozenge

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent</th>
<th>Amount soluble in mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled Water</td>
<td>2.46</td>
</tr>
<tr>
<td>2</td>
<td>0.1N HCl (1.2 pH)</td>
<td>7.89</td>
</tr>
<tr>
<td>3</td>
<td>6.8 pH Phosphate Buffer</td>
<td>4.46</td>
</tr>
<tr>
<td>4</td>
<td>7.2 pH Phosphate Buffer</td>
<td>2.68</td>
</tr>
</tbody>
</table>

Table 1: Saturated Solubility Studies of Amoxicillin Trihydrate in Different Dissolution Media

<table>
<thead>
<tr>
<th>S.No</th>
<th>Ingredients</th>
<th>Lozenges formulations(in mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
<td>AMF₁</td>
</tr>
<tr>
<td>1.</td>
<td>Amoxicillin</td>
<td>250</td>
</tr>
<tr>
<td>2.</td>
<td>Ginger</td>
<td>—</td>
</tr>
<tr>
<td>3.</td>
<td>Tulsi</td>
<td>—</td>
</tr>
<tr>
<td>4.</td>
<td>PEG 4000</td>
<td>—</td>
</tr>
<tr>
<td>5.</td>
<td>MCC</td>
<td>—</td>
</tr>
<tr>
<td>6.</td>
<td>Stevia</td>
<td>—</td>
</tr>
<tr>
<td>7.</td>
<td>Silica gel</td>
<td>—</td>
</tr>
<tr>
<td>8.</td>
<td>Citric acid</td>
<td>—</td>
</tr>
<tr>
<td>9.</td>
<td>Acacia</td>
<td>—</td>
</tr>
<tr>
<td>10.</td>
<td>Total weight(in mg)</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 2: Composition of lozenges formulations

* [one lozege containing 250mg of amoxicillin trihydrate]

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formulations was found to be linear with first order rate constant. The $R^2$ values of all lozenge formulations were in the range of 0.87 to 0.93. The rate of drug release of lozenge formulations was found to be linear with Hixon Crowell rate constant. The $R^2$ values of all lozenge formulations were in the range of 0.812 to 0.951. FTIR studies were performed for pure drug, polymers and optimized lozenge formulations. In FTIR studies, the groups in pure amoxicillin trihydrate and optimized formulations were having similar fundamental peaks and pattern. This indicates that there were no drug-excipient interactions in the formulations. Taste was masked effectively for the formulation AMF3 & AMF4 prepared by melt and mold technique. Antimicrobial activity studies were performed for different lozenge formulations. AMF2 formulation showed greater Zone of inhibition. This may be due to synergistic antimicrobial effect of Amoxicillin Trihydrate, Tulsi and Ginger. Accelerated stability studies were carried out for some selected lozenge formulations. There was no significant change observed in physical parameters such as weight uniformity, friability, hardness, and drug content. Drug release from the lozenges after storage at

<table>
<thead>
<tr>
<th>S.No</th>
<th>Lozenge formulation</th>
<th>Weight uniformity (g/loz)</th>
<th>Weight of Individual lozenge</th>
<th>Hardness (kg/cm²)</th>
<th>Drug content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMF₁</td>
<td>1.25 ± 0.3</td>
<td>1.28</td>
<td>1.1±0.3</td>
<td>247 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>AMF₂</td>
<td>1.42 ± 0.1</td>
<td>1.44</td>
<td>1.2±0.3</td>
<td>247 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>AMF₃</td>
<td>1.42 ± 0.3</td>
<td>1.44</td>
<td>1.1±0.1</td>
<td>248± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>AMF₄</td>
<td>1.42 ± 0.1</td>
<td>1.44</td>
<td>1.1±0.2</td>
<td>247± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>AMF₅</td>
<td>1.42 ± 0.2</td>
<td>1.44</td>
<td>1.3±0.1</td>
<td>248± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>AMF₆</td>
<td>1.42 ± 0.2</td>
<td>1.44</td>
<td>1.1±0.2</td>
<td>248± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.No</th>
<th>Lozenge Formulations</th>
<th>$T_{90}$</th>
<th>DE 30 %</th>
<th>First order</th>
<th>Hixoncrowell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$K$ (min⁻¹)</td>
<td>$R^2$</td>
</tr>
<tr>
<td>1</td>
<td>AMF₁</td>
<td>30</td>
<td>35</td>
<td>0.0257</td>
<td>0.857</td>
</tr>
<tr>
<td>2</td>
<td>AMF₂</td>
<td>17.5</td>
<td>40</td>
<td>0.0278</td>
<td>0.879</td>
</tr>
<tr>
<td>3</td>
<td>AMF₃</td>
<td>9</td>
<td>70</td>
<td>0.0310</td>
<td>0.857</td>
</tr>
<tr>
<td>4</td>
<td>AMF₄</td>
<td>12</td>
<td>63.3</td>
<td>0.0898</td>
<td>0.910</td>
</tr>
<tr>
<td>5</td>
<td>AMF₅</td>
<td>19</td>
<td>56.6</td>
<td>0.0794</td>
<td>0.859</td>
</tr>
<tr>
<td>6</td>
<td>AMF₆</td>
<td>14</td>
<td>56.6</td>
<td>0.0776</td>
<td>0.875</td>
</tr>
</tbody>
</table>
different conditions remained unaltered and found to be quite stable. The formulations subjected to Accelerated Stability studies were also characterized by FTIR, the results revealed that there was no major interaction between the drug and polymers.

**Future scope of work**

Formulation AMF3 and AMF4 was found to release the drug at slower rate and is suitable for preparing as lozenges. Further studies can be focused on the Amoxicillin Trihydrate by using newer natural ingredients, polymers and matrices and even in the combinations of these. Investigation can be extended by employing newer techniques for the preparation of lozenges. In-vivo pharmacokinetic and dynamic studies can be performed on a suitable animal model.

**Conclusion**

The present study showed that it is possible to formulate the amoxicillin trihydrate as lozenges. Of all the soft lozenge formulations, the formulations AMF3 and AMF4 containing 1000 and 900 mg of PEG 4000 and 100 and 200 mg of MCC showed the slow release of the drug i.e. up to 45 minutes. The formulations containing Stevia as sweetener are also stable after storage. Hence the natural sweeteners such as Stevia can be considered as an alternative replacement for the artificial sweeteners in the preparation of lozenges. AMF2 formulation showed greater Zone of inhibition. This may be due to synergistic antimicrobial effect of Amoxicillin Trihydrate, Tulsi and Ginger. Accelerated stability studies were carried out for some selected lozenge formulations. There was no significant change observed in physical parameters such as weight uniformity, friability, hardness, and drug content. Drug release from the lozenges after storage at different conditions remained unaltered and found to be quite stable.

**Acknowledgements**

The authors express their gratitude to Apotex Pharma Ltd, Bangalore for providing the gift sample. The authors are thankful to the management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur for providing the facilities to carry out the research work.

**References**


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Abstract

In view of the fluoride induced toxic effects on human health; an effective and simple biosorption method was developed for fluoride laden groundwater using zirconium alginate beads. The various parameters affecting the batch adsorption in fluoride spiked groundwater such as initial fluoride concentration (2.5-10 mg/L), residence time (10-120 min) and pH (5, 7, 9) were studied. The fluoride concentration in the solutions was measured by spectrophotometry at 550 nm using in-house developed zirconyl-xylenol orange complex reagent. The biosorbent was characterized with techniques such as X-ray diffraction (XRD), Fourier transform infrared Spectroscopy (FTIR), scanning electron microscopy (SEM) and energy dispersive X-ray fluorescence (EDX). At pH 5, 7 and 9; fluoride removal of 90.5, 46.6 and 88.8% by the biosorbent was noted in groundwater spiked with 10 mg/L of fluoride, within 30 min. Thus, the zirconium alginate beads were found to be potential biosorbents for remediation of groundwater contaminated with fluoride. Further studies are going on to find out the reusability of the developed biosorbent at field conditions. The biosorption mechanism was derived from sorption studies, FTIR, SEM and EDXRF techniques.

Key words: alginate, bioremediation, biosorption, fluoride, groundwater, zirconium

Introduction

The fluoride ion has been classified as one of the major contaminants in drinking water and its pollution in groundwater has been recognized as a severe problem worldwide. It enters into the water resources via dissolution of geogenic minerals and anthropogenic discharge of effluents from metal plating, semiconductor, glass and ceramic manufacturing; coal, uranium and aluminium mining; and fertilizer industries. The World Health Organization (WHO) has laid down a maximum permissible limit of 1.5 mg/L for fluoride in drinking water. Though minute level of fluoride is beneficial for humans in the range of 0.5-1.0 mg/L, the excess intake is detrimental leading to dental and skeletal fluorosis. More than 70 countries of the world including India, Sri Lanka, China, Thailand, Turkey, Iraq, Iran, Afghanistan, Japan, South Africa, USA, Mexico, Argentina, Jordan, Egypt, Libya, Algeria, Sudan and Kenya have drinking water supplies naturally contaminated with fluoride. An elevated fluoride concentration in ground and drinking waters has been reported in nearly 20 states of India such as Rajasthan, Telangana, Andhra Pradesh, Karnataka, Maharashtra, Gujarat, Madhya Pradesh, Bihar, Jharkhand, West Bengal, Haryana, Tamil Nadu, Assam, Punjab, Chhattisgarh, Kerala, Uttar Pradesh, Odisha, Delhi, Jammu and Kashmir. Numerous techniques such as ion exchange, precipitation, membrane
separation are generally used for the elimination of fluoride from water. However, the precipitation method is limited by the requirement of additional treatment, sludge generation, high cost and presence of residual aluminium. The ion exchange and membrane separation techniques are energy intensive and expensive (Paudyal et al., 2013; Velazquez-Jimenez et al., 2015). As an alternative, adsorption of fluoride from groundwater using a natural, renewable, functional group enriched biopolymer, alginate was attempted, as a simple and cost effective methodology.

The biopolymer alginate is a naturally occurring anionic polysaccharide produced by a variety of brown algae and the bacteria *Pseudomonas aeruginosa* and *Azotobacter*. The important characteristics of the alginate are its (i) renewability, (ii) biodegradability, (iii) non toxicity (iv) gelation, (v) generally recognized as safe (GRAS) status and (vi) low cost. It is widely used as a thickening agent in textile printing, emulsifier, stabilizer, thickener and gelling agent in food and disintegrating agent in pharmaceutical industries. It is also used as a micro-encapsulation agent for the production of insoluble artificial seeds in plant tissue culture, entrapping/immobilizing agent for enzymes, as a haemostatic agent in skin wound dressings, an impression-making material in prosthetics and dentistry, as a hydrogel in bioengineering research, fish feed binder and as a sustained drug delivery system (Hitoshi et al., 2002; Lee and Mooney, 2012).

In view of the fluoride induced toxic effects on human health; an effective and simple biosorption method was developed for the fluoride laden groundwater using zirconium alginate beads. The effect of initial fluoride concentration, residence time and pH on batch adsorption of fluoride in spiked groundwater was investigated. The fluoride concentration in the solutions was measured by spectrophotometry at 550 nm using in-house developed zirconyl-xylenetol orange complex reagent. The biosorbent was characterized with X-ray diffraction, Fourier transform infrared spectroscopy, scanning electron microscope and energy dispersive X-ray fluorescence techniques.

**Materials and methods**

Zirconium oxychloride octahydrate (Loba Chemie, Mumbai, India), sodium alginate (Sigma, Bengaluru, India), calcium chloride dihydrate, hydrochloric acid and xylenetol orange (SD Fine, Mumbai, India) of AR grade were used. A 100 mg/mL certified fluoride calibration standard solution (Thermo Scientific Orion 940907) traceable to National Institute of Standards and Technology (NIST) reference material was used for working standard solution preparation. The ultrapure water with a resistivity of 18.2 MΩm obtained from Elga Purelab Flex 3 ultrapure water polishing unit (High Wycombe, England) was used for all the experiments.

**Preparation of zirconium alginate beads:** The zirconium alginate beads were prepared according to the reported method with minor modifications (Huo Yakun et al., 2011). A 3% (w/v) of homogenous sodium alginate polymer solution was prepared in ultrapure water by continuous overnight stirring at room temperature. A 10 mL of sodium alginate solution was dropped into 30 mL of 200 mM zirconium oxychloride solution, using a 5 mL micropipette tip under continuous stirring. The drops were turned out into beads of 4-5 mm diameter in zirconium oxychloride solution and the beads were further soaked in the same solution for 24 h for completion of gelation process. Then, the beads were washed thoroughly with ultrapure water for removal of unreacted zirconium ions (Fig. 1).

**Characterization of groundwater:** The groundwater samples were collected from the bore well of National Centre for Compositional characterization of Materials (NCCCM), Hyderabad, India. The collected water was analyzed for physicochemical parameters including pH, conductivity and total dissolved solids using Elico water quality analyzer PE 138 (Hyderabad, India). The fluoride content in the groundwater samples was measured by

Aruna
spectrophotometric method, based on zirconyl-xylenol orange complex reagent. The metal indicator dye, xylenol orange forms an orange coloured complex with zirconium ions (Zr⁴⁺), which is decolourized via the dissociation of the complex by the fluoride ions leading to the formation of colourless zirconium fluoride. The reagent zirconyl-xylenol orange was prepared by mixing 0.01% (w/v) of the dye with 0.04% (w/v) of depolymerized zirconium solution in 20% (v/v) of HCl. The reagent and the sample was added in 1:4 volume ratio, i.e. 1 mL of the reagent was mixed with 4 mL of the sample solution and the resulting colour of the mixture was determined by recording the absorbance at 550 nm (Cabello-Tomas and West, 1969; Ružička et al., 1966; S. V. Rao et al., 2002). The fluoride concentration in the sample solutions were estimated based on the calibration plots obtained with aqueous standards (Fig. 2). The concentration of various metals such as calcium, magnesium, zinc, barium, iron; manganese, copper, lead and cadmium in groundwater was determined using Jobin Yvon Horiba JY-2000 inductively coupled plasma-optical emission spectrometer (ICP-OES) (Longjumeau, France) and Analytik Jena AG Contra AA 700 continuum source-electro thermal atomic absorption spectrometer (CS-ETAAS) (Jena, Germany), respectively. The concentration of anions such as sulphate, chloride and phosphate in groundwater was analyzed with Dionex ICS-3000 ion chromatography system (Sunnyvale, USA) employing IonPac AG20 guard and IonPac AS20 analytical columns. The total heterotrophic bacteria in groundwater were enumerated using nutrient agar medium. The total coliforms and *Escherichia coli* were concurrently determined with HiCrome coliform selective agar medium (Table 1).

**Biosorption studies:** The fluoride uptake studies were carried out in batch mode by equilibrating 0.3 g of the zirconium alginate beads with 10 mL of groundwater spiked with different fluoride concentrations (2.5-10 mg/L) at various pH (5, 7 and 9) conditions under continuous mixing. A control experiment was also done by equilibrating 0.3 g of zirconium alginate beads with 10 mL of groundwater, with no fluoride addition. The samples were collected at specific intervals (10-120 min) and the supernatants were collected after centrifugation. The centrifugates were analyzed

![Fig. 1](image1.png) The digital photograph of zirconium alginate biosorbent beads.

![Fig. 2](image2.png) The calibration plot showing the absorbance at 550 nm vs. fluoride concentration. Inset: Solution colour at various fluoride levels.

A renewable source for the biosorption of fluoride from contaminated ground water
for fluoride levels using an Analytic Jena AG Specord 200 Plus UV-visible spectrophotometer (Jena, Germany). The amount of adsorbed fluoride per unit weight of the zirconium alginate biosorbent was quantified with the following equation

\[ q = \frac{(C_0 - C_e)V}{m} \]  

Where, 
\( q \) = Amount of fluoride adsorbed per unit weight of biosorbent (mg/g)  
\( C_0 \) = Initial fluoride concentration (mg/L)  
\( C_e \) = Equilibrium fluoride concentration (mg/L)  
\( V \) = Solution volume (L)  
\( m \) = Dry weight of the biosorbent (g)

In triplicate the biosorption experiments were done and the data were represented as mean (±) standard deviation (SD).

**Characterization of biosorbent:** The Labconco Freezone 4.5L Plus benchtop cascade freezedry system (Kansas City, USA) was used for lyophilization of native and fluoride sorbed biosorbents. The Bruker Optics TENSOR 27 FTIR spectrometer (Ettlingen, Germany) was employed for recording the IR spectra of the lyophilized powders at 1000-4000 cm\(^{-1}\). The Rigaku, Ultima IV diffractometer (Tokyo, Japan) was utilized for X-ray diffraction analysis at 40 kV and 30 mA using monochromatic Cu K\(\alpha\) radiation (\(\lambda = 1.5406 \) Å). The intensity data was recorded for the powdered biosorbents at a 2\(\theta\) range of 35-70° and scan rate of 1°/min. The lyophilized powders of native and fluoride sorbed adsorbent were carbon sputter coated and visualized under Zeiss EVO 18 Research scanning electron microscope (Carl Zeiss Microscopy Ltd., Cambridge, UK) at an accelerating voltage of 20 kV. The energy dispersive X-ray fluorescence spectra of the biosorbents were collected with x-act (Oxford Instruments Analytical Ltd., High Wycombe, UK) spectrometer fitted with silicon drift detector, running at 20 kV.

**Results and discussion**

The adsorption method is preferred for the removal of fluoride ions from groundwater due to its low cost, design simplicity, flexibility, selectivity and efficiency. It can be exploited in a developing country like India, where renewable, cheap and locally available adsorptive resources are abundant in comparison with expensive, synthetic adsorption media used in industrialized countries (Velazquez-Jimenez et al., 2015). In literature, alginate beads doped with Fe (III) (Velazquez-Jimenez et al., 2015), Al (Zhou et al., 2014), hydrous ferric oxide (Sujana et al., 2013) and La (Huo Yakun et al., 2011) were used as adsorptive materials for fluoride removal from water. In this context, zirconium alginate beads were prepared from renewable, natural biopolymer alginate for the removal of fluoride from groundwater.

**Fluoride uptake studies:** The obtained zirconium alginate beads were spherical in shape and size ranged from 4-5 mm. The spherical shape of the beads also favours its application in continuous flow fixed bed columns (Li et al., 2013). The capability of zirconium alginate beads towards fluoride uptake in ground water was studied at varying fluoride ion concentration (2.5-10 mg/L), solution pH (5, 7 and 9) and residence time (10-120 min) in batch mode. The critical parameter in determining the fluoride adsorption is the solution pH. The pH effect on fluoride uptake at an initial fluoride concentration of 2.5 mg/L at varying reaction time of 10-120 min is shown in Fig. 3. The fluoride uptake of 75.7 and 72.5% was noted at pH 5 and 9, respectively in comparison with 47.5% at pH 7, within 10 min. At pH 5 and 9 and 30 min of reaction, the uptakes were 87.7 and 95.8%, respectively indicating the near saturation of adsorption. While at pH 7, the uptake is linear and increased with an increase in reaction time and showed an uptake of 63.1% only at 30 min. Thus, the data suggests that fluoride ion adsorption is rapid at pH 5 and 9, in comparison with neutral pH of 7. Similar trend was observed at a concentration of 5 mg/L, with corresponding uptake of 87.3, 88.7 and 63.1% at pH 5, 9 and 7,
respectively (Fig. 4). The uptakes of 90.4, 92.5
and 50.6% were noted at 7.5 mg/L of fluoride, at
pH 5, 9 and 7, respectively (Fig. 4). At an elevated
concentration of 10 mg/L of fluoride, the respective
uptakes were 90.5, 88.8 and 46.6% at pH 5, 9
and 7 (Fig. 5). The pH dependent biosorption is in
concurrence with earlier fluoride removal study with
lanthanum alginate bead (Huo Yakun et al., 2011).
At 120 min, the removal values were found be 98,
98 and 93% at pH 5, 9 and 7, respectively at 10
mg/L of fluoride. At these conditions, the fluoride
uptake values were 5.1, 5.1 and 4.8 mg/g,
respectively. These values are comparable with
earlier fluoride biosorption study carried out using
hydrous ferric oxide doped alginate beads (8.9
mg/g) (Sujana et al., 2013) and alginate
bioencapsulated nano-hydroxyapatite composite
(3.8 mg/g) (Pandi and Viswanathan, 2014). In the
current report, it is significant to note that the
fluoride removal was achieved at variable

Fig. 3 The time dependent fluoride uptake by the
biosorbent at pH 5 and varying initial fluoride
concentration.

Fig. 4 The time dependent fluoride uptake by the
biosorbent at pH 7 and varying initial fluoride
concentration.

Fig. 5 The time dependent fluoride uptake by the
biosorbent at pH 9 and varying initial fluoride
concentration.

Fig. 6 The XRD pattern of biosorbents sodium
alginate, zirconium alginate and fluoride sorbed
zirconium alginate.
groundwater pH. In addition, the biosorption was successful at physicochemical conditions of groundwater such as total dissolved solids (750 mg/L) and in the presence of cations including Ca (143 mg/L), Mg (23.4 mg/L), Mn (293.8 μg/L), Cu (130.1 μg/L); and anions such as Cl⁻ (108 mg/L), SO₄²⁻ (100 mg/L) and NO₃⁻ (14.8 mg/L).

**Biosorbent characterization and mechanism:**

The biosorbent zirconium alginate was characterized by an array of techniques before and after uptake of fluoride from groundwater.

**X-ray diffraction (XRD):** The XRD pattern of sodium alginate, zirconium alginate and fluoride sorbed zirconium alginate is shown in Fig. 6. For sodium alginate, the three characteristic peaks were noted at 13.5 °, 22.9 ° and 39 ° indicating the semi crystalline structure (Fontes et al., 2013; Helmiyati and Aprilliza, 2017; Huo Yakun et al., 2011). But in the case of zirconium alginate and fluoride sorbed zirconium alginate, the one of the peaks disappeared and the other became less intense, implying a change in the crystal structure. The amorphous nature of the zirconium alginate sorbent facilitates a better access to fluoride and zirconium is known for its affinity towards fluoride (Huo Yakun et al., 2011).

**Fourier transform infrared spectroscopy (FTIR):** The IR spectra of zirconium alginate before and after fluoride adsorption are indicated in Fig. 7. The zirconium alginate exhibits characteristic absorbance peaks at 3135, 1751, 1639, 1402, 1319, 1276, 1127, 1027, 827 and 778 cm⁻¹. The broad peak at 3135 corresponds to OH functional groups of the biopolymer. The peak at 1751 cm⁻¹ could be accounted to carbonyl stretch of carboxylic acids. The dominant peaks at 1639 and 1402 cm⁻¹ could be ascribed to symmetric and asymmetrical stretches of carboxylate groups, respectively. The peaks observed at 1276 and 1127; 1027 and 827 cm⁻¹ attribute to C-O stretches of polyols, uronic and mannuronic acids, respectively.

While, after fluoride uptake the zirconium alginate shows peaks at 3136, 1827, 1657, 1402, 1319, 1276, 1124, 1011, 827 and 778 cm⁻¹. After adsorption, a major shift in the absorbance peaks of carboxylate and C-O-C groups was noted from 1751 to 1827, 1639 to 1657; and 1027 to 1011.
cm\(^{-1}\), respectively. Thus, it indicates a coordination interaction between Zr\(^{4+}\) and F\(^{-}\), based on hard soft acid-base theory. The inductive electronic attraction effect of halogen atom with metal ions brings a shift in the peaks of carboxylate and C-O-C groups (Zhou et al., 2014). It is known that the positively charged Zr\(^{4+}\) ions at the surface of alginate attracts the negatively charged fluoride ions via electrostatic interaction (Vijaya et al., 2011). Thus, the results confirm the electrostatic and coordination interaction mechanism in defluoridation of groundwater (Sujana et al., 2013; Vijaya et al., 2011; Zhou et al., 2014).

**Scanning electron microscopy (SEM) and Energy dispersive X-ray fluorescence (EDX):**

The surface and morphological characteristics of zirconium alginate before and after fluoride uptake was visualized using SEM (Fig. 8). Before fluoride adsorption, the biosorbent was porous, flaky and fibrillar in nature, which aid in efficient diffusion of fluoride ions into the matrix and adsorption rate. Upon fluoride uptake, significant morphological and structural changes such as shrinkage, enhanced roughness and compactness of biosorbent were noticed, indicating that the fluoride adsorption is indeed a surface phenomenon (Helmiyati and Aprilliza, 2017; Huo Yakun et al., 2011; Kora et al., 2016; Li et al., 2013; Qiusheng et al., 2015; Vijaya et al., 2011; Zhou et al., 2014). The energy dispersive X-ray fluorescence (EDX) spectra of native and fluoride sorbed zirconium alginate are shown in Fig 9. The presence of fluoride in the biosorbent was confirmed from the shown peak in the spectra, along with other elements such as Zr, Cl, C, O, Na, K, Ca etc after fluoride uptake (Pandi and Viswanathan, 2014; Sujana et al., 2013; Swain et al., 2013).

**Conclusions**

A simple biosorbent based method for the fluoride removal from groundwater was developed using zirconium alginate beads. At pH 5, 9 and 7; 98, 98 and 93% removal was noted in 10 mg/L fluoride spiked groundwater within 120 min. The zirconium alginate beads could be used as a potential biosorbent for the remediation of fluoride contaminated groundwater. Further detailed

**Tab. 1** The compositional analysis of collected groundwater.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.0 ± 0.05</td>
</tr>
<tr>
<td>Conductivity (mS/cm)</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>Total dissolved solids (mg/L)</td>
<td>750 ± 10.4</td>
</tr>
<tr>
<td>Calcium (mg/L)</td>
<td>143 ± 5</td>
</tr>
<tr>
<td>Magnesium (mg/L)</td>
<td>23.4 ± 1.3</td>
</tr>
<tr>
<td>Zinc (mg/L)</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Barium (mg/L)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Iron (mg/L)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Manganese (μg/L)</td>
<td>293.87 ± 6.4</td>
</tr>
<tr>
<td>Copper (μg/L)</td>
<td>130.1 ± 15.2</td>
</tr>
<tr>
<td>Lead (μg/L)</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>Cadmium (μg/L)</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td>Chloride (mg/L)</td>
<td>108 ± 5.7</td>
</tr>
<tr>
<td>Fluoride (mg/L)</td>
<td>1.14 ± 0.07</td>
</tr>
<tr>
<td>Sulphate (mg/L)</td>
<td>100 ± 1.5</td>
</tr>
<tr>
<td>Nitrate (mg/L)</td>
<td>14.86 ± 0.9</td>
</tr>
<tr>
<td>Total heterotrophic bacteria (CFU/mL)</td>
<td>8.5 × 10^{2}</td>
</tr>
<tr>
<td>Total coliforms (CFU/100 mL)</td>
<td>0</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (CFU/100 mL)</td>
<td>0</td>
</tr>
</tbody>
</table>

A renewable source for the biosorption of fluoride from contaminated ground water
studies are envisaged on fluoride sorption characteristics of the biosorbent in column mode to find out its suitability and reusability at field conditions.

Acknowledgement

The author would like to thank Dr. Athyala Christian Sahayam, Head, Bulk Analysis Section (BAS) and Dr. Sunil Jai Kumar, Former Head, NCCCM/BARC for their constant encouragement and support throughout the study.

References


A renewable source for the biosorption of fluoride from contaminated groundwater
Abstract

In this study, we aimed to investigate the possible cardioprotective effect of a water-soluble polysaccharide (LEP) from the leaves of Lycium europaeum against cisplatin (CP)-induced heart injury in mice model.

Mice (31 ± 2 g) were divided into four groups (n=6) as follows: Group (1) control, Group (2) LEP alone (100 mg/kg, p.o.), Group (3) CP (10 mg/kg, i.p.), Group (4) LEP + CP and Group (5) Atorvastatin, 10 mg/kg + CP. To assess the efficacy of LEP treatment against CP-induced cardiotoxicity, lipid peroxidation, biochemical parameters and histopathological examinations were conducted.

CP administration induced severe heart injuries and elevated lipid peroxidation levels. However, LEP pretreatment reduced cardiovascular diseases, which has been evinced by amelioration in parameters such as triglyceride, total cholesterol, lactate dehydrogenase, low-density lipoprotein-cholesterol and high-density lipoprotein-cholesterol. In addition, cardiac indexes, atherogenic indexes, and coronary artery indexes were significantly improved in Group (4) when compared to CP intoxicated animals. Moreover, LEP administration to CP-treated mice obviously mitigated the malondialdehyde level when compared to Group (3). The histopathological observations also demonstrated that LEP pretreatment significantly restored the damage induced by CP.

These results advised that LEP improved myocardial injury and could be recommended as a potential candidate for the development of new cardioprotective agents.

Key words: Lycium europaeum; Cisplatin; Cardioprotective; Polysaccharide; Heart injury.

Introduction

Cisplatin (CP) is one of the most used chemotherapeutic agents for the treatment of manifold cancer types (1). However, CP usage has been seriously associated with many undesirable side effects including nephrotoxicity (2) and cardiotoxicity (3). Indeed the cytotoxicity of CP lies in its interaction with mitochondrial DNA, which causes cardiomyopathy, bradycardia and arrhythmia (4, 5). Many studies have confirmed that cisplatin-induced cardiotoxicity stimulates the generation of free radicals (6, 7). Therefore, research has been developed to mitigate the side effects of CP without perturbing its antitumor effects. Previous studies indicated that antioxidants including L-carnitine and silymarin were able to circumvent the cardiotoxic effect associated with CP intoxication in mice (7). Numerous researches have already revealed the beneficial effects of natural plant polysaccharides in the management of many illnesses. For instance, polysaccharides extracted from Nitraria retusa have demonstrated potential hepatoprotective and cardioprotective effects in experimental mice (8). Cao et al. (9) proved the

In vivo cardioprotective effect of a polysaccharide
protective effect of polysaccharides extracted from *Astragalus membranaceus* against doxorubicin-induced cardiotoxicity.

In our previous study, we extracted and characterized polysaccharides from the leaves of *Lycium europaeum* (LEP) (2). Results showed that LEP was composed of 77.59% of carbohydrates and 2.25% of protein. The hepatoprotective and nephroprotective effects of LEP in mice were determined. The findings revealed that the administration of LEP at 100 mg/kg had a protective effect against hepatotoxicity and nephrotoxicity induced by CCl₄ and cisplatin, respectively. However, the effects of LEP on CP-induced cardiotoxicity have never been elucidated. Herein we examine the protective effect of LEP against CP-induced oxidative stress and cardiovascular disorders in mice.

**Materials and methods**

**Chemicals and preparation of extract:** Cisplatin was purchased from Merck (Darmstadt, Germany). Chemicals products and assay kits were purchased from Sigma Chemical Co. (St. Louis, MO). The polysaccharide from *Lycium europaeum* (LEP) was prepared according to our previous publication (2).

**Cisplatin-induced cardiotoxicity in vivo**

**Experimental Animals:** Male Swiss albino mice (31 ± 2 g, 12 weeks old) were got from the animal house of the Faculty of Science Sfax, Tunisia.

Healthy animals were housed in cages, kept under standard laboratory conditions; temperature was 22 ± 2 °C with 40% humidity and allowed free access on balanced diet (according to NRC 1995) (10) and drinking water. Experimental tests were accomplished in concordance with standard ethical guidelines for laboratory animal use and care as explained in European Community Guidelines.

**Experimental Setup:** Cardiotoxicity in mice was induced by intraperitoneal injection (i.p.) of cisplatin (CP, 10 mg/kg, i.p.) on the 5th day (6).

In this study a total of 30 healthy animals were randomly divided into four groups (n=6 each) as follows:

- Group 1 (control) mice received NaCl (0.9%, i.p) for ten days; group 2 (LEP) mice were orally administered LEP (100 mg/kg,) for 10 successive day (2); group 3 (CP) mice were intoxicated after a single injection of CP (CP, 10 mg/kg, i.p.) on the fifth day, group 4 (LEP+ CP) animals were treated with LEP at 100 mg/kg, i.p. for 5 successive days before and after a single dose of CP on the fifth day and group 5 (ATV + CP) animals received standard drug (Atorvastatin, 10 mg/kg) for 5 successive days before and after a single dose of CP on the fifth day.

Twenty four hours after the last treatment, animals were sacrificed. Blood samples were collected from the left ventricular of heart. The heart was directly removed, weighed and dissected into two halves, one for biochemical assays and the other for histopathological analysis.

**Body weight and heart weight:** The body weights (BW) of mice were measured at the beginning of the treatment and on the day of sacrifice and the gains (%) of BW were calculated. The hearts of animals were isolated and weighed to determine the absolute and relative weights.

**Determination of cardiac biomarkers:** Biomarker enzymes for cardiac function including lactate dehydrogenase (LDH), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol were estimated using an auto-analyzer (Roche Cobas C 311, Germany).

Indexes including cardiac (CI), atherogenic (AI) and coronary artery (CAI) were calculated by these formulas: (1); (2); (3)

\[
(1) \ Cl = \frac{TC}{HDLc}
\]

\[
(2) \ AI = \frac{TC-HDLc}{HDLc}
\]

\[
(3) \ CAI = \frac{LDLc}{HDLc}
\]

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Heart biomarkers enzymes including creatine kinase: CK-NAC and creatine kinase-MB: CK-MB were evaluated using commercial reagent kits from Biomaghreb (Tunisia) according to the manufacturer’s protocol.

**Lipid peroxidation assay (LPO):** The LPO in tissue homogenates was evaluated using the procedure of Ohkawa et al. (11). Heart tissues were homogenized in phosphate-buffered saline (0.1 M; pH 7.4) with an Ultra Turrax homogenizer. Next, the mixture was centrifuged at 1500 g for 15 min to give the supernatant which will be used to investigate LPO. The absorbance of each tested group was recorded at 532 nm and the results were expressed as the MDA content.

**Histopathological analysis:** Heart tissues were kept 48 h into formalin solution (10%), then dehydrated in gradual concentrations of alcohol from 70 to 100% and embedded in paraffin. Finally, paraffin was cut at 5 μm thickness, stained with hematoxylin-eosin and analyzed under a light microscope.

**Statistical analysis:** Data were expressed as means and standard deviation of means (±SD) and analyzed using the SPSS software program (PASW Statistics 18.0). All data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey’s test.

**Results**

**Effects of LEP treatment on the body and heart weights:** The effects of LEP on CP-intoxicated mice are summarized in Table 1. No changes were observed in the body and heart weights of mice treated with normal saline solution and with LEP alone. However, these parameters in CP-intoxicated mice were significantly decreased compared to the control animals. In the same way, the body weight gains and the relative heart weight were significantly reduced in CP-intoxicated mice as compared to the control group. A significant protection was recorded in the LEP+CP treated group compared with the CP group.

**Effects of LEP treatment on biochemical parameters:** The cardioprotective potentials of LEP at 100 mg/kg were assessed in terms of their aptitude to decrease the levels of TC, TG, LDL and LDH. Cisplatin treatment produced a significant rise in the levels of these biomarkers when compared to the normal group (Table 2). In contrast, the pretreatment of mice with LEP or either ATV significantly reduced the cardiotoxic effect of cisplatin as compared to CP-treated group. Table 2 also displayed a significant decrease in the level of HDL and increase in the ratio of AI, CI and CAI in groups treated with cisplatin in comparison to the control group. Also, significant elevations for CK-NAC and CK-MB levels, when compared to control group. However, these disorders were clearly restored in mice pretreated with LEP when compared to the CP-induced cardiotoxicity group. Interestingly, treatment of CP-intoxicated mice with Atorvastatin restored the normal serum levels of all aforementioned parameters.

**Effects of LEP treatment on heart lipid peroxidation:** As shown in Table 2, the treatment with cisplatin enhanced the level of MDA (3.82 nmol/mg protein) in comparison to the control group (1.17 nmol/mg protein). However, pretreatment of mice with LEP before CP injection resulted in a significantly down in MDA level as compared to the intoxicated mice (1.88 nmol/mg protein, versus 3.82 nmol/mg protein, p< 0.05).

**Effects of LEP treatment on heart histopathological tissue:** The cardiac tissues in the control and LEP groups showed normal histoarchitecture of the heart (Fig.1A and B). A large and irregularly shaped hypertrophic myocardial fiber and hemorrhages were observed in the cisplatin-treated group (Fig.1C). However, LEP pretreatment (100 mg/kg) prior to cisplatin administration ameliorated heart histological tissue (Fig.1D) as compared to the control group.

**Discussion**

Oxidative stress is one of the main mechanisms involved in the pathogenesis of heart

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failure. The use of phytochemicals as a therapeutic approach for cardiovascular disease is gaining care worldwide. In the present investigation, the protective effect of LEP was examined on oxidative stress and myocardial damage in cisplatin-induced cardiotoxicity in mice. CP treatment alone resulted in body and heart weight changes. These changes can be attributed to gastrointestinal toxicity and the minimization in the ingestion of food (12). Moreover, CP treatment may lead to hypertrophy of the heart tissue due to the increased water content, dematous intramuscular space (13). This result is similar to reports by Afsar et al. (14). However, LEP supplementation in the CP-treated mice restored the weight changes probably by preventing oxidative stress-induced cell death.

Results revealed that the administration of cisplatin to healthy mice caused a marked cardiac

![Figure 1](image-url). Photomicrographs of myocardial tissues in the control and experimental treated mice (hematoxylin–eosin, ×200). The control group and group treated with *Lycium europaeum* (LEP, 100 mg/kg, b.w.) showing normal cardiac muscle fibers. Cisplatin treated group (10 mg/kg, b.w.) showing large inflammatory cells infiltration, myocardial cells necrosis, separation of cardiac myofibrillar. LEP + cisplatin co-treated group showing normal myocardial arrangement and few inflammatory cells.

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dysfunction that was evident by increased plasma CK-NAC and CK-MB levels.

The observed troubles look to be linked to the alteration in the membrane permeability and/or the death of myocardial cells, as results of cisplatin intoxication, leading then to the release of cytosolic contents into the systemic circulation, which was in harmony with previous studies (15). Treatment of CP-intoxicated mice by either LEP or Atorvastatin significantly restored the normal plasma levels of all aforementioned parameters.

Data of the present work revealed that cisplatin treatment is associated with the alterations in lipid profile which was evident by the increase in the levels of TC, TG and LDL and the decrease in the HDL level. These abnormalities were in agreement with previous finding demonstrating hyperlipidemia in animals treated with cisplatin (6). Disorders in the lipid profile have been demonstrated for increasing the risk of myocardial infarction. Moreover, the high level of LDL-C in plasma can contribute to the formation of atherosclerosis plaques due to the lipid deposition on the arterial wall. The treatment with LEP significantly restored these parameters to normal levels in comparison to CP-treated groups, thereby reducing the risk of cardiovascular diseases. Our previous studies have confirmed that LEP protects against the toxic effects of CP via its powerful antioxidant potential (2). Likewise, pretreatment with Atorvastatin (10 mg/kg) attenuated the cardiac damage caused by CP (16).

Cardiac, atherogenic and coronary artery indices are predictive indicators of cardiac illnesses. In this study, mice treated with CP exhibited a profound increase in AI, CI and CAI as compared to the normal ones. Pretreatment with LEP (100 mg/kg, i.p.) followed by CP administration revealed a significant improvement in cardiovascular risk indices and subsequently minimized the possibility of cardiovascular disease occurrence (17). Similar effects have been recorded in previous work from our lab on polysaccharides extracted from Nitraria retusa fruits (8).

It has been demonstrated that cisplatin generates reactive oxygen species and stimulates lipid peroxidation that leads to the cardiotoxicity (18). In our study, the injection of CP resulted in oxidative damage, detected by the increase in the cardiac content of MDA. Our results are in line with those of Afsar et al. (14) that demonstrated an increase in lipid peroxidation through the high cardiac MDA levels in response to cisplatin administration. Normalization of the cardiac MDA levels in experimental groups pre-treated with LEP, suggesting its cardioprotective effect. All the above results were supported by histopathological investigations. Significant histological changes were observed in the heart of mice treated with CP including atrophic myocardial fiber and hemorrhages, which might be due to the generation of ROS that provoked damages in the histoarchitecture of cardiac tissues (14). Same toxic influences were detected using other toxic elements on cardiac tissue such as the isoproterenol (19). However, pre-treatment of mice with LEP alleviated the precedent histoarchitecture disruptions.

Conclusions
The present study demonstrated for the first time that LEP improved cardiovascular performance against cisplatin-induced heart injuries in mice. The effect is associated with a restoration of the malondialdehyde level, the histoarchitecture of cardiac tissues and the enhancement of biochemical parameters, the cardiac index and the atherogenic index. Collectively, these findings demonstrated that LEP might be a potential therapeutic medicine for the treatment of cardiovascular disease by reducing CP induced myocardial injuries.

Acknowledgments
We greatly appreciate the technical support provided by the Unit of common services, Faculty of Sciences Gafsa, Tunisia.

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### Table 1. Effect of CP and LEP treatment on body weight and heart weight.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Body weight (BW)</th>
<th>Heart weight (HW)</th>
<th>Relative weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial (g)</td>
<td>Final (g)</td>
<td>Gains (%)</td>
</tr>
<tr>
<td>Control</td>
<td>31.4 ± 1.07</td>
<td>37.4 ± 0.51</td>
<td>16.03 ± 3.03</td>
</tr>
<tr>
<td>LEP</td>
<td>30.8 ± 1.22</td>
<td>38.6 ± 1.07</td>
<td>20.15 ± 3.68</td>
</tr>
<tr>
<td>CP</td>
<td>31.0 ± 1.15</td>
<td>32.0 ± 1.15</td>
<td>3.12 ± 0.10</td>
</tr>
<tr>
<td>LEP + CP</td>
<td>30.6 ± 1.26</td>
<td>39.8 ± 0.78</td>
<td>23.13 ± 2.15</td>
</tr>
<tr>
<td>ATV + CP</td>
<td>30.4 ± 1.42</td>
<td>38.2 ± 0.78</td>
<td>20.38 ± 4.18</td>
</tr>
</tbody>
</table>

Data are expressed as means SD for six mice in each group.

* Significant difference at p < 0.05 and *** at p < 0.001 between control and CP treated group.

### Table 2. Effect of the administration of CP and LEP on heart lipid peroxidation and plasma biochemical parameters in the different experimental groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment (mg/kg)</th>
<th>Control</th>
<th>LEP</th>
<th>CP</th>
<th>LEP + CP</th>
<th>ATV + CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td></td>
<td>1.17 ± 0.08</td>
<td>1.35 ± 0.10</td>
<td>3.82 ± 0.03</td>
<td>1.88 ± 0.04</td>
<td>1.84 ± 0.04</td>
</tr>
<tr>
<td>CK-NAC (U/L)</td>
<td></td>
<td>188.74 ± 3.53</td>
<td>180.79 ± 2.66</td>
<td>294.19 ± 5.33</td>
<td>212.16 ± 2.25</td>
<td>201.06 ± 1.91</td>
</tr>
<tr>
<td>CK-MB (U/L)</td>
<td></td>
<td>209.76 ± 1.22</td>
<td>212.14 ± 6.91</td>
<td>317.83 ± 2.90</td>
<td>247.56 ± 5.47</td>
<td>227.9 ± 3.25</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td></td>
<td>582.71 ± 5.26</td>
<td>575.01 ± 10.82</td>
<td>832.66 ± 9.75</td>
<td>609.87 ± 6.05</td>
<td>595.1 ± 6.82</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td></td>
<td>2.13 ± 0.03</td>
<td>2.26 ± 0.08</td>
<td>3.12 ± 0.07</td>
<td>2.44 ± 0.05</td>
<td>2.24 ± 0.12</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td></td>
<td>0.93 ± 0.01</td>
<td>1.04 ± 0.05</td>
<td>1.67 ± 0.03</td>
<td>1.18 ± 0.05</td>
<td>1.12 ± 0.08</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td></td>
<td>0.50 ± 0.01</td>
<td>0.49 ± 0.01</td>
<td>0.98 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td></td>
<td>1.50 ± 0.04</td>
<td>1.46 ± 0.01</td>
<td>1.13 ± 0.05</td>
<td>1.35 ± 0.02</td>
<td>1.39 ± 0.03</td>
</tr>
<tr>
<td>CI</td>
<td></td>
<td>1.42 ± 0.02</td>
<td>1.54 ± 0.05</td>
<td>2.75 ± 0.15</td>
<td>1.81 ± 0.00</td>
<td>1.61 ± 0.09</td>
</tr>
<tr>
<td>AI</td>
<td></td>
<td>0.42 ± 0.02</td>
<td>0.54 ± 0.05</td>
<td>1.75 ± 0.15</td>
<td>0.81 ± 0.00</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>CAI</td>
<td></td>
<td>0.33 ± 0.02</td>
<td>0.33 ± 0.00</td>
<td>0.86 ± 0.01</td>
<td>0.47 ± 0.03</td>
<td>0.36 ± 0.03</td>
</tr>
</tbody>
</table>

Data are expressed as means SD for six mice in each group.

* Significant difference at p < 0.05 and *** at p < 0.001 between control and CP treated group.

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References


In vivo cardioprotective effect of a polysaccharide
Abstract

Human dependence on plants, especially for medicinal purpose can be dated back to centuries and this reliance continues till date for various reasons such as minimal cost and easy availability in nature. Ocimum sanctum, commonly known as ‘Holy basil’ is a sacred plant with immense medicinal significance. The present review aims to summarize the holy basil research works and its therapeutic potentials. Juice extracted from the holy basil is used to cure a wide array of ailments such as cold, headache, fevers, eye and mouth disorders and holds anti-stress activity. It controls the blood cholesterol levels, thereby aids in curing cardiac disorders. Also, the plant extracts were proven to possess anti-oxidant, anti-helminthic, anti-inflammatory, anti-diabetic and anti-microbial activities. Specifically, the holy basil extracts possess anti-cancerous properties besides radioprotective properties. In addition, Ocimum sanctum products are also helpful to maintain immune levels. Hence, the wide spectrum of medicinal benefits and the consistent increase in demand for plant-based medicines, we have been encouraged to discover further therapeutic values of the sacred holy basil.

Key words: Holy basil, Anti-inflammatory, Anti-cancer, Anti-diabetic, Anti-oxidant, Anti-microbial

Introduction

Since time immemorial plants were given utmost importance in the field of medicine besides serving basic necessities such as food, shelter and clothing (1). The reliance continues till date for several reasons such as minimal cost, easy availability in nature and short life span of synthetic drugs. Scholars of all ages gathered huge knowledge on medicinal plants through trial and error methods which is in practice till date (2, 3). Specifically, they worked on medicinal plants and mentioned their nomenclature, healing properties and provided complex description of the interpretation of medicines. The knowledge of medicinal plants and their therapeutic properties urges the isolation of certain industrial chemicals which are useful to cure various diseases (4). The world health organization (W.H.O) estimates that a large number of people especially in developing countries still rely on traditional plant-derived drugs since they are more economical (5). In contrast, only 1% of phytochemical composition has been investigated amongst a global luxurious gene pool of plant kingdom particularly using leaves, stem, root, flowers and seeds of medicinal plants (2). Several plants are yet to be investigated for their chemical composition and medicinal properties. Generally, medicinal herbs are subjected to
rigorous chemical analysis and the bioactive components found are isolated depending on their composition. Later these compounds are evaluated through pharmacological methods using appropriate procedures, thereby increasing the utility and efficiency of these drugs (6). The pharmacological properties of medicinal plants vary with the composition of metabolites which are unique to individual species. Long back, Wink (7) reported that the feasibility of research extension in medicinal plants is possible through identifying the active principles of secondary metabolites. Parallel to the allopathic drug system, usage of about 2000 or more diversified medicinal plant species in ayurvedic and unani system of medicine has been recorded (8, 9). In addition, the plant medicines need not be sold in the form of powders, instead direct crude extracts of roots, stems and leaves are the best alternatives. Number of indigenous drug industries were established recently which supply either ready made medicines for direct use or partially processed raw material for the preparation of prescriptions (10).

**Ocimum sanctum** Linn. (syn. *Ocimum tenuiflorum*) commonly known as 'Holy basil' or 'Tulsi' belongs to the family Lamiaceae, which possess a number of therapeutic compounds (4). Holy basil is found in most parts of tropical and semitropical regions in the world. In India, holy basil is grown in all areas due to its countless medicinal benefits as well as its integration into the daily lives of the people, especially its use in religious ceremonies that made it sacred (6). Apart from Indian system, it is a vibrant medicinal plant in other systems of medicine in most of the countries (9, 11). Normally, the solvents used to extract the holy basil are ethanol, methanol, benzene etc. (12). Also, extracts of holy basil (EHB) are used either alone or in combination with other herbal plants to cure various diseases (13). Apart from medicinal purpose, the powdered holy basil is used to make aromatic beverages along with other herbal products in certain countries (14). The genus *Ocimum* has a number of species depending on the region it grows and possess comparable chemical composition (15, 16). More than 150 *Ocimum* species with numerous cultivars are distributed in different regions and grow up to 6000 feet above sea level (3, 16, 17). Some of the known important species of *Ocimum* includes *O. americanum*, *O. angustifolium*, *O. basilicum*, *O. carnosum*, *O. gratissimum*, *O. minimum*, *O. serratum* etc. Holy basil is used since thousands of years for its healing properties and is often called the queen of herbs as mentioned in *Charaka Samhita* written by a legend in the field of Indian medicine (14). In the present review, several medicinal properties of holy basil are discussed in detail using different case studies.

**Bioactive therapeutic components of holy basil:** Holy basil is one of the most reliable resources in the area of medical science since ancient times. Although number of compounds are isolated and characterized, however further investigations are necessary to investigate remaining compounds of holy basil and related *Ocimum* species (11, 12, 17, 18). One of the active constituents of *O. sanctum* is eugenol (2-methoxy-4-(2-propenyl) phenol), an allylbenzene class of volatile compound which is a major part in its essential oil (4). Holy basil is reported to possess ursolic acid (1S, 2R, 4aS, 6aR, 6aS, 6bR, 8aR, 10S, 12aR, 14bS) -10-hydroxy-1,2, 6a, 6b, 9, 9, 12a-heptamethyl-2, 3, 4, 5, 6, 6a, 7, 8a, 10, 11, 12, 13, 14b-tetradecahydro-1H-picene-4a-carboxylic acid) and monoterpenoid phenol namely carvacrol (5-isopropyl 2-methyl phenol), as important medicinal components (Fig. 1). In addition, holy basil possess rosmarinic acid ((2R)-3-(3,4-dihydroxyphenyl)-2-{{[(2E)-3- (3, 4-dihydroxyphenyl) prop-2-enyl] oxy} propanoic acid), linalool (3,7-dimethylocta-1,6-dien-3-ol), caryophyllene (1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo [7.2.0] undec-4-ene, terpinene-4-ol, (+) α cadinene, 3-careen, eugenol methyl ester and alpha humulene (14).

It also possess important flavonoids such as apigenin (4',5,7-trihydroxyflavone), orientin, vicenin-2, luteolin and other phytoconstituents
including cirsimaritin, α-pinene, cervacrol, methylchavicol, isothymusin, isothymon, palmitric acid, vallinin, galic acid, ocimumoside A, ocimumoside B and ocimarin (9, 11, 12, 18). Different chemical compounds mentioned above exhibited mild to severe action against various diseases.

Functions of chemical components and pharmacological uses: In ancient days, without knowing much phytochemistry, extract of holy basil was used to treat a number of diseases based on the success rate, which was mentioned in ayurveda and naturopathy (4, 6). Initially, holy basil has been used in the treatment of headache (14, 15). Later, by identifying the therapeutic compounds through advanced technology, it was established as a powerful medicinal plant. Extracts of holy basil prepared with various solvents are used to cure several diseases including common colds, cough, headache, mouth and eye disorders, diabetic, heart disorders, cancer, inflammation, other microbial diseases and different fevers as mentioned in Figure 2 (9).

Fig. 1: Some of the therapeutic components of Ocimum

Due to the presence of eugenol and linoleic acid in leaf extracts, holy basil is an excellent remedy for cough, cold and various fevers including malaria and dengue (6, 19). Similarly due to the presence of eugenol, EHB is also used to cure bronchitis. Leaf powder of Tulsi mixed with sandalwood paste is an excellent remedy against heat, headache and allied diseases (4). Holy basil is used to treat regular illnesses, fungal and

Fig. 2. Medicinal properties of holy basil. Tulsi is majorly used as anti-cancer, anti-inflammatory, anti-oxidant, anthelmintic, anti-diabetic, anti-microbial as well as other medicinal activities.

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bacterial infections because of essential oil (20, 21). Also, Tulsi is used to repel insects in stored grains by mixing dried leaves due to the presence of volatile oil (22). In addition, the extract of this plant is used as repellent to control mosquitoes that often act as vectors in spreading different fever causing pathogens (23, 24). Due to the presence of ocimumosides A and B, holy basil is one of the best anti-stress agents (18). Ursolic acid, a pentacyclic triterpene acid is used to stabilize the body from certain pains and is also helpful in recovering from stress (13, 25). According to several studies, chewing holy basil leaves leads to notable changes in salivary pH and enhances the bicarbonate concentration in salivation due to the presence of essential oil, terpenes, terpenoids which includes eugenol, ursolic acid, carvacrol etc. (26). Holy basil’s essential oil possess eugenol, caryophyllene and linalool that plays a key role in curing certain diseases including anti-microbial activities (6, 9). Moreover, major components of holy basil are responsible for preventing dental problems (27, 28, 29). These compounds also improves the metabolic function, levels of immunity, lowers the stress, holds anti-oxidant property by reducing liver lipid synthesis, enhances insulin secretion and reduces inflammation (4, 6, 9, 25, 30). Singh (16) also noticed that the anti-inflammation property of Tulsi due to the presence of chemical compounds such as eugenol, apigenin, rosmarinic acid, cirsimaritin etc. Phytochemicals of holy basil alters the metabolic function, cures obesity related problems and exhibits anti-oxidant property due to the presence of eugenol, isothymusin, isothymonin, rosmarinic acid etc. (31). Mainly the presence of linoleic acid in holy basil is extremely beneficial to treat certain diseases including inflammation and inhibited various cancer cell lines proliferation (16, 32). Ursolic acid down regulates the proliferation and arrested cell cycle at G1 and G0 phases which induces apoptosis in cancer cells and suppresses the activities of nuclear factor (NF)-8B activation (9, 33). Moreover eugenol, ursolic acid, linoleic

**Fig. 3: Holy basil effect on various cancer cells:** Holy Basil inhibits proliferation of different cancer cell lines and induces apoptosis by enhancing the ROS and Caspase 3,8 activity and down regulates tumor formation on human fibrocarcinoma and Lewis lung carcinoma models.

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acid, betulinic acid, (-)-rabdosin, vicenin-2, orientin etc., were actively involved in anti-cancerous activities (11, 12, 34).

**Therapeutic properties of holy basil:** The present work offers detailed information on potential therapeutic values of the holy basil. The phytochemical analysis and pharmacological investigations of *O. sanctum* has helped in the treatment of cold, fever and various respiratory ailments such as bronchitis, asthma, influenza and cough (35). Anti-dengue and anti-typhoid activities of Tulsi were recently demonstrated by Tang et al (19) and Mandal et al. (23). Leaf extracts of holy basil elevates the salivary pH and curbs the acidic environment in mouth and stomach thereby, beating ulcers (4, 36). Phytoconstituents of holy basil also prevents dental issues such as caries, plaque, bad breath etc., by mainly targeting *Streptococcus mutans* which are responsible for tooth decay (27, 28, 37). In Ayurvedic medicine, an important poly herbal eye drop extract is prepared with Ocimum, Caesalpina, Jasminum and Cynodon for the treatment of eye disorders and conjunctival congestion in rats as well as other animals. Holy basil is used to prepare eye drops along with Triphala, which is useful to cure cataract, glaucoma, chronic conjunctivitis and other eye-related diseases (11, 35). Further, essential oil obtained from distillation of *O. sanctum* leaves is used extensively in the pharmaceutical industry majorly in skin cream preparation (38). Tabassum and Hamdani (39) prepared a moisturizer to cure acne (skin disease) using ethanolic extract of holy basil (EEOS) as one of the ingredients along with *Andrographis paniculata*, *Glycyrrhiza glabra*, *Azaadiracta indica* and green tea extracts. Due to the presence of rich anti-oxidants, ethanolic and aqueous extract of holy basil fastens wound healing in rat skin (40). Gupta et al. (18) isolated ocimumosides A and B from the leaves of holy basil, which are implicated as anti-stress agents. Treatment with Tulsi leaves in albino rats with oxidative stress showed increased superoxide dismutase and reduced glutathione indicating its anti-stressor activity (41).

Myocardial infarction (MI), also known as heart attack is one of the primary reasons for fatality due to the shortage of blood flow to heart and its muscles. Effect of hydroalcoholic extract of holy basil at different doses was investigated against isoproterenol-induced myocardial infarction in rats and found significantly reduced levels of glutathione (GSH), superoxide dismutase (SOD) and lactate dehydrogenase (LDH). The extract also inhibited lipid peroxidation with the maximum cardioprotective effect at 50mg/kg dose (42). Recently, Kavitha et al. (43) proved the relation between inflammation and myocardial infarction using Tulsi extract. Blood clotting within vessels is one of the main diseases, which leads to blood flow issues. Extract of Tulsi exhibits thrombolytic potential along with curcuma, azadirachta and anacardium (44). It was studied that extract of *O. sanctum* leaves exhibited the reversible anti-fertility effect due to the presence of key components such as eugenol (45, 46). Albino rats treated with holy basil extract (250 mg/kg body weight) exhibited decreased total sperm count and sperm motility as well as an increase in abnormal sperms. Withdrawal of treatment led to regaining of normal conditions within two weeks (46).

The anthelmintic or anti-parasitic activity of the essential oil of holy basil was evaluated by model *Caenorhabditis elegans* and proved that eugenol is the predominant component in putative anti-helminthic principle (47, 48). Crude aqueous and hydroalcoholic extract of *O. sanctum* showed anthelmintic activity against ovine gastrointestinal nematodes in sheep and no deleterious effect was found indicating that holy basil is safe to use (49). Various extracts of holy basil exhibited anti-inflammatory activity in different animal models (50). Eugenol, cirsilineol, cirsimaritin, apigenin and rosmarinic acid are the main compounds involved in anti-inflammatory activity (9, 35). An ethyl acetate root extract of *O. sanctum* was proved to be the best anti-inflammatory agent in carrageenan-induced paw edema model (51).

Anti-diabetic and anti-hyperlipidemic effects of hydroalcoholic extracts of holy basil have
been evaluated using diabetic rats induced by streptozotocin (STZ) and nicotinamide (52). This experiment predicts the biological activities of holy basil constituents and significantly exhibits the anti-diabetic effect compared to standard drug glibenclamide. In diabetic induced rats, oral administration of extract of Tulsi decreased the blood sugar content. Further, effects of holy basil on aldose reductase activity could help in reducing the complications of diabetes such as cataract, retinopathy etc. (53). Hypoglycemic condition was noticed when normal rats were fed with fructose and later treated with extracts of holy basil for 30 days (54). It was noticed that leaves of Tulsi reduced glucose and cortisol in serum of corticosteroid-induced diabetes mellitus. Gholap and Kar (55) unravel the possible mechanism of glucose-lowering activity of \textit{O. sanctum} in male mice. In another study, Chattopadyay [56] also observed the reduction of blood sugar levels with oral administration of extract of Tulsi in streptozotocin-induced diabetic rats.

Cancer is one of the most life threatening diseases across the world for several reasons. Statistically, it was estimated that about 1,665,540 new cases of cancer were expected to be diagnosed by the American Cancer Society 2019 (57). Various groups of drugs work in different ways to fight against cancer cells and shrink tumors (58). Apart from synthetic drugs, herbs and sea weeds are also useful for cancer remedy (59). The findings of Kim et al. (60) exhibited that ethanolic extract of \textit{O. sanctum} inactivates matrix metalloproteinase-9, stimulated anti-oxidant enzymes, inhibited proliferation of Lewis lung carcinoma (LLC) cells and reduced the volume of tumors in mice. Sridevi et al. (32) also reported that alcoholic root extract of Tulsi inhibited cell proliferation and induced apoptosis through enhancing intracellular reactive oxygen species (ROS) in human non-small cell lung carcinoma cell line (NCl-H460). Recently, Utispan et al. (61) proved the anti-invasive effect of ethanolic leaf extracts of holy basil on head and neck squamous cell carcinoma (HNSCC) cell line by attenuating matrix metalloproteinase (MMP) activity. In vitro anti-cancer activity of holy basil has been demonstrated using human fibrosarcoma (HFS) cells. The ethanolic extract of \textit{O. sanctum} influences lipid peroxidation and decrease the rate of solid tumor formation in tumor-bearing mice (62). Extract of holy basil enhanced anti-tumor activity through down regulating apoptosis regulator BAX and enhancing caspase-3 and cytochrome c which in turn induces apoptosis in forestomach of N-methyl N'-nitro-N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis model (63). \textit{Ocimum sanctum} inhibits proliferation of non-small cell lung carcinoma A549 and induces apoptosis by activating caspase-3 (64). Extract of Tulsi reduces the damage of DNA in normal cells and induces apoptosis in SiHa cancer cells (65). Ehrlich ascites carcinoma (EAC) mice treated with leaf extract of holy basil decreased the haemoglobin content and increased the WBC (white blood cells), which implicit that this sacred plant has high anti-neoplastic activity (66). Ethanol and acetone leaf extracts of \textit{O. sanctum} inhibits the growth of A549 lung cancer cell line but aqueous extract does not show any anti-cancer activity (67). Flegkas et al. (68) isolated rabdosiin \textit{(terpenoid)} from \textit{O. sanctum} leaves, which induced apoptosis in HCT-116, MCF-7 and SKBR3. It also exhibited reduced cytotoxicity to normal human peripheral blood mononuclear cells. However, orientin from holy basil is non cytotoxic to human cancer cells HepG2 (69). Major cultivars of \textit{O. sanctum} including ‘rama’ and ‘krishna’ induced apoptosis in mouth epithelial carcinoma cells (KB) (70). These results indicate that most of the holy basil possess anti-tumor properties. Figure 3 represents the anti-cancerous activity of \textit{O. sanctum} in detail.

Thyroid diseases are generally diagnosed using radioactive iodine therapy that causes structural and functional damage to the salivary glands as a result of lipid peroxidation by reactive oxygen species (71). Aqueous extract of \textit{O. sanctum} and its components such as orientin and vicenin significantly decreased the lipid

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peroxidation, leading to membrane protection of salivary glands, thereby reducing the incidence of thyroid cancer (72). Thus, holy basil can be used as potential candidate for radioprotection against iodine induced salivary gland damage (73).

Several laboratory studies proved that *O. sanctum* protects the body against damage caused by toxic chemicals by increasing the level of antioxidant enzyme activities (74). These extracts initiate the anti-oxidant enzymes which protect organelles and radical membranes that are caused due to lack of oxygen and other toxic substances (75). The phytochemicals in Tulsi extract are rich in anti-oxidants, which can be used as an effective preservative in the food industry. In cadmium treated albino rats, oral administration of hydroalcoholic extract of *O. sanctum* protect cadmium damage by decreasing lipid peroxidation and by up regulating the superoxide dismutase, catalase and glutathione peroxidase activities (76). In sodium fluoride treated rats, chemical compound such as carvacrol of holy basil protect the liver tissue from fluoride toxicity and also modulates the anti-oxidant enzymes (77). Hydroalcholic extract of *O. sanctum* down regulates the lipid peroxidation and inhibits DNA damage and reactive oxygen species generated by mitochondrial membrane in SH-SY5Y human neuronal cells (78).

Phytochemicals of holy basil have been evaluated for its radioprotective effects using different models (14, 79). Leaves of *O. sanctum* consist certain compounds such as cirsilineol, isothymusin, apigenin, rosmarinic acid, eugenol etc. were exhibited the diverse pharmacological effects, including radioprotective activity (80). In tumor-bearing animals, holy basil and other plant flavonoids were shown to have a protective effect towards normal tissue during radiation, thereby permitting the application of higher dose of radiation in order to control severe tumors (81, 82).

*Ocimum sanctum* acts as an important immunomodulator and exhibits great impact on human health (83). The immune system is a complex network of organs, tissues, cell types and proteins that orchestrate collectively to protect the host from bacterial, fungal, viral and other parasitic infections as well as from tumor growth (84). Immunomodulaor (biological and chemical agent) can interfere and alter the functioning pattern of immune response either through immunopotentiation, immunosuppression or immunological tolerance. Leaf extract of Tulsi increases the production of antibody by stimulating the humoral immune response (85, 86). Vaghasiya et al. (87) compared the alcoholic and aqueous extracts of holy basil in immune modulatory activity. It was reported that the oral administration of aqueous extract of *O. sanctum* in wister albino rats stimulates the antibody production as compared to untreated samples and also enhanced the production of white blood cells, red blood cells and hemoglobin (88).

Benzene and methanolic extracts of holy basil combined with ampicillin inhibits the *E. coli*, *P. aeruginosa* and *S. aureus* cultures when compared to ampicillin alone (89). Similarly, alcoholic, aqueous and chloroform extracts of *O. sanctum* leaves exhibit adverse effects on *E. coli*, *S. aureus*, *P. aeruginosa* and *S. typhimurium*. Compounds of the holy basil are known to inhibit the growth of both gram-positive and gram-negative bacteria (21). Fixed oil of *O. sanctum* demonstrates anti-bacterial effect towards *B. pumilus*, *S. aureus* and *P. aeruginosa* (90). Also, aqueous extract of holy basil showed a high zone of inhibition towards *E. coli*, Klebsiella and *S. aureus* when compared to ethanolic extract (91). Methanolic extract of Tulsi leaves damages the growth of *V. cholerae* cells and show MBC (minimal bactericidal concentration) at 0.5-3.0 mg/ml concentration (92). Apart from above mentioned medicinal properties, ethanolic extract of Tulsi exhibited normal wound healing and dexamethasone-depressed healing properties in albino rats (93). The extract significantly stimulated the wound breaking strength, made wound epithelialize faster as compared to the control. In addition, dried powder or fresh leaf extracts of Tulsi are often consumed in various forms such as herbal Dakshayani et al
tea, which reduces the formation of stones in the kidney (3).

Need of biotechnological tools to enhance the use of holy basil: Conservation of ancient natural treasure such as holy basil and the capability to utilize it in a sustainable manner are essential for the well being and continued survival of humans (94). There are a number of constraints for systematic collection of elite Ocimum and its cultivars, which include variations in edaphic and climatic factors, low percentage of seed set and seasonal dormancy (4, 6). Moreover, future work on Tulsi may require more finesse to exploit additional medicinal benefits (9). The application of biotechnological techniques such as in vitro propagation and genetic engineering could prove to be a great benefit for low cost production of these plants with additional secondary metabolites (95). Advantage of these techniques lead to overcome seasonal dormancy. In vitro clonal propagation of holy basil enables large scale production of therapeutically high value taxa for commercialization and sustainable utilization in the industrial sector. In addition, it would be more beneficial if tablets could be prepared with powdered holy basil for commercial purpose.

Conclusion

Ocimum sanctum generally known as holy basil holds a set of potential phytochemicals, which possess high therapeutic properties for various diseases including cancer. Extracts of Ocimum have certain specificity in the treatment of cold, cough, various kinds of fevers including malaria, dengue and respiratory ailments such as bronchitis, asthma, influenza that spread extensively during particular seasons. Extracts of holy basil also work as an anti-inflammatory, anti-cancer, anti-thyroid, anti-diabetic, anti-oxidant, anti-microbial agent and is also involved in anthelmintic activities. Tulsi juice has the capacity to cure mouth and teeth infections, eye disorders as well as heart and vascular disorders. It also possesses radioprotective property and can balance the immunity levels in the human body. Hence, studies on this sacred plant can be warranted as extremely beneficial for the human community in the field of medicine. Because of anti-viral property, Tulsi extract may be helpful to cure different viral diseases which need to confirm in near future. Likely, this review reports the therapeutic properties of holy basil in detail.

Conflict of interest

The authors declared that they have no conflict of interest.

Acknowledgements

The authors are thankful to Dr. K. Paramesh and Dr. D. Vajaranad, Yogi Vemana University, India for their technical help.

References


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Abstract
Agriculture is the source of GDP for any country. Effort is being made to analyze the trend of contributors at India and global with identification of major/top research areas, publishing journals, fund sources, organizations, researchers; group authors and cited papers in the index databases of Clarivate Analytics from 2014-2019. India is leading globally in the area of smart agricultural, more paper published in the stream of computer science and engineering. After exploration of this area it is observed that India maintains its growth at global level from last six years. Pure research work performed mainly in environmental science, S & T and biodiversity conservation. National agencies are funding more at national and International level. French National Centre for Scientific Research (CNRS) and Indian Council of Agricultural Research (ICAR) are doing much effort to promote smart agriculture at abroad and home respectively. IEEE (Institute of Electrical and Electronics Engineers) and IOP (Institute of Physic) are top group authors. Our study reveals that Indian papers did not get much citation.

Key words: Smart agriculture, Mitigation, Biotic Stress, Abiotic stress, Conservation, Natural resources, Biodiversity

Introduction
Agriculture plays a crucial role in the economy of developing countries, and provides the main source of food, income and employment to their rural populations. According to FAO (2000), it has been established that the share of the agricultural population in the total populace is 67% that agriculture accounts for 39.4% of the GDP and that 43% of all exports consist of agricultural goods. So, world is making serious efforts to make agriculture as a progressive industry by addressing the following challenges: (i) the shortage of agricultural labors; (ii) shortage of agricultural produce; (iii) reduce harvest and post harvest loses; and (iv) monitoring the real time agricultural environment. Such efforts essentially mean complete utilization of resources and the potential of rural areas through technological innovation. The state-of-art robot technology, information and communication technology (ICT), cloud-enabled CLAY-MIST measurement (CMM) index, wireless sensor network, optical sensors, use of Internet of Things (IoT), Message Queing Telemetry Tracking (MQTT), and other cutting-edge technologies are used to promote smart agricultural practices. Such smart practices help in achieving economic progress and establishing sustainable agro-ecosystems. In this context, in alignment with the theme of “Smart Agriculture”, we have made an attempt to assess the academic output in this subject area. Indexed publications that emerge from research works serve as an Advanced Package Tool (APT) to measure academic output in any given area.

Smart agriculture: India
**Objectives**

- To find the academic output of India in field of smart agriculture only;
- To explore the area of smart agriculture in the border term;
- To analyze the Indian output Vs Globally;
- To identify the top research areas, funding agencies, journals, leading organization authors and joint authors of India and at global level;
- To identify the top and hot papers on the basis of citations at national and International Level.

**Methodology**

We choose the topic of “smart agriculture” as well as focus on some important agricultural areas i.e. “Mitigation of abiotic/biotic stresses”, “Conservation of natural resources with biodiversity” for our data search. We looked for records under the “Topics” for six year period from 2014 to 2019 in the Web of Science (Wos) Core Collection, an authentic source of research publications and widely used index database of Clarivate Analytics. We used Smart Agricult*, Mitigation with Abiotic/Biotic stress, Conservation with natural resource* and Conservation with biodiversity as the main keywords for the search in Citation Indices - Science Citation Index (SCI), Social Science Citation Index (SSCI), Conference Proceedings Citation Index of Science (CPCI-S) and Social Sciences and Humanities (CPCI-SSH), from WoS. The data has been downloaded on September 22, 2020 from the databases. We restricted the further search on the basis of documents viz., Articles, Reviews, Proceeding Papers, Editorial Materials, Meeting Abstracts, Early Access and Data Paper. We used intermediary excel sheets or files for the depth analysis of data and for the expected outcomes.

**Analysis and Findings**

*India’s Academic Output in Smart Agriculture:* Initially, we focused on smart agriculture in respect of India so limited our search around our choose topic “smart agriculture” with restricted document types, then we received 605 records globally and found India position is first with 119 records. When we explore these records more and refined then we received 82 pure Indian papers, we observed that most of research work published in the form of Articles and proceedings papers i.e. pure research work performed in the zone of Computer Science, Engineering and Telecommunication and the main funding agency is Department of Science (DST). The leading national Journal is Indian Journal of Agricultural Sciences. Jat ML and Kumar A are main Indian contributors. The IEEE (Institute of Electrical and Electronics Engineers) is the main professional association.

**Comparison- India’s Academic Output at Global Level:** Our study revealed interesting rather encouraging facts (Table 1). In “smart agriculture” there are 605 papers published during this period globally, and India leads with 119 papers. Similarly, in the focal themes, “Mitigation of abiotic/biotic stresses” India leads in total number of publications and hold first rank at globally. We noticed good number of publications on conservation aspects. In conservation of natural resources there were 3188 records and India ranks is 7th with 171 records. We received 25136 records globally in the area of “Biodiversity” in which India holds 17th rank with 713 publications.

Further, we included all the search keywords and made single search with the help of advance search module of WoS, to find out the combined result (Table 2). In our finding, we received 28045 documents through WoS and India’s Rank is 14th with 995 documents. We observed that in 2014, India position is 16th with 110 documents (3592 is global output) and in 2019, India position is 12th with 217 documents (5775 is global output). It is observed the percentage growth is maintained by India at International Level. The upward chart of academic output of (India Vs Globally) illustrated in Figure 1 & 2, which exhibit the demand and interest of researcher towards innovation in form smart agriculture. The H index of Indian paper is 37 and average citations per item are 8.76, citing articles are 7589, sum of times cited is 8723 and
We examine further more data and filtered records on the basis of document type and observed that the academic output produced mainly in the form of Articles, Review papers and Proceeding papers i.e. pure research performed in the stream of smart agriculture (Figure 3).

For knowing the top research areas we again filtered the data and analyzed that Environmental Sciences Ecology, Science Technology and Biodiversity Conservation are the main areas in which more research works performed at national and International Level (Figure 4).

We also curious about to know that the leading research and peer viewed journal in India and Global Level, so we continuing the filtering without self-citation citing 7321 and sum of times cited is 8243.

Table-1. Smart Agriculture: publications trend in last six years (2014-2019).

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Table-2. India at International Level (Year 2014-2019).

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<td>2019</td>
<td>217</td>
<td>5775</td>
<td>3.757</td>
</tr>
</tbody>
</table>

Smart agriculture: India
Fig. 1. Shows academic and research growth of India Vs Global; (a) India & (b) Global.

Fig. 3. Illustrated the publication trend of India Vs Global in six years; (a) India & (b) Global.

Fig. 4. Details of top ten research areas, in which most of the research performed in India Vs Global; (a) India & (b) Global.

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procedure and the illustrated result (Figure 5) display that Plos One, Biological Conservation with Biodiversity and Conservation are the top most journals those were published the number of paper in the field of smart agriculture. In the case of India, Current Science and tropical ecology are the main leading journals.

Funding is very important factor for initiative, progress and completion of any kind of research, hence it is very essential to know the funding agencies. For this we filtered and sorted our data after the few cross-checking criteria. The result (Figure 6) demonstrated that Department of Science & Technology (DST), University Grants Commission (UGC) and Indian Council of Agricultural Research (ICAR) are the top three funding agencies, which shows the focus of Indian Government on the smart agriculture (because Indian economic based on agriculture from ancient era) i.e. government interested in advanced and ICT based smart agriculture. At global level National Council for Scientific and Technological Development (CNPQ), National Science Foundation (NSF) and National Natural Science Foundation of China (NSFC) are top most funding organizations, those are worked to promote smart agriculture at international level i.e. word move towards formal kind of agriculture to smart agriculture which based on hi-tech machinery, technologies and tools.

From the above result our approaches moves toward the working organizations and we would like to explore the institutions, those are performed the researches in the zone of smart agriculture; again we filtered and sorted our data. After some validating criteria we received the details of top eight organizations of India and abroad (Figure 7). The Indian Council of Agricultural Research (ICAR), Council of Scientific and Industrial Research (CSIR) and Indian Institute of Technology
(IIT System) are the main organizations; those are produced smart agriculture based innovative researches. In global scenario the Centre National De La Recherche Scientifique (CNRS), University of California System and Chinese Academy of Sciences are produced most significant works for encouragement of smart agriculture globally.

We were last filtered the data, to identify the top most researcher or authors of India and aboard (Figure 8) as well as we to identify the top most group authors and individual researchers of India (Figure 9). We looked that there are three Indian authors - Reddy CS, JHA CS and Kumar A those contributed in more or equal to 20 research papers and two authors Possingham HP and Lindenmayer DB contributed in more than 100 research papers globally. In the case of group authors, IEEE (Institute of Electrical and Electronics Engineers), ACM (Association for Computing Machinery) and IOP (Institute of Physics) are the top most group author at the International level.

Our last sorting is used to analyze the citation of papers because citation is used to know the utility of any kind of research. Therefore, we sorted our result on the ground of received citation in respect of India and Globally. The Indian papers received less citation in comparison to global level papers.
Table 3. List of highly cited papers (green colour denoted the Indian papers and blue for global).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Authors</th>
<th>Article Title</th>
<th>Journal Name</th>
<th>Publication Year</th>
<th>Cited Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pascual U et al.</td>
<td>Valuing nature's contributions to people: the IPHES approach</td>
<td>Current Opinion in Environmental Sustainability</td>
<td>2017</td>
<td>332</td>
</tr>
<tr>
<td>2</td>
<td>Anderson-Teixeira K et al.</td>
<td>CTFS-ForestGEO: a worldwide network monitoring forests in an era of global change</td>
<td>Global Change Biology</td>
<td>2015</td>
<td>243</td>
</tr>
<tr>
<td>5</td>
<td>Jit R K</td>
<td>Seven years of conservation agriculture in a rice-wheat rotation of Eastern Gangetic Plains of South Asia: Yield trends and economic profitability</td>
<td>Field Crops Research</td>
<td>2014</td>
<td>110</td>
</tr>
<tr>
<td>6</td>
<td>Moemu K K et al.</td>
<td>Abiotic Stress Responses and Microbe-Mediated Mitigation in Plants: The Omics Strategies</td>
<td>Frontiers in Plant Science</td>
<td>2017</td>
<td>105</td>
</tr>
<tr>
<td>1</td>
<td>Ripple W J et al.</td>
<td>Status and Ecological Effects of the World's Largest Carnivores</td>
<td>Science</td>
<td>2014</td>
<td>1198</td>
</tr>
<tr>
<td>2</td>
<td>Dirozo R et al.</td>
<td>Deforestation in the Anthropocene</td>
<td>Science</td>
<td>2014</td>
<td>1188</td>
</tr>
<tr>
<td>3</td>
<td>Prum S J et al.</td>
<td>The biodiversity of species and their rates of extinction, distribution, and protection</td>
<td>Science</td>
<td>2014</td>
<td>1029</td>
</tr>
<tr>
<td>4</td>
<td>Haddad N M et al.</td>
<td>Habitat fragmentation and its lasting impact on Earth's ecosystems</td>
<td>Science Advances</td>
<td>2015</td>
<td>992</td>
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<tr>
<td>5</td>
<td>Ceballos G et al.</td>
<td>Accelerated modern human-induced species losses: Entering the sixth mass extinction</td>
<td>Science Advances</td>
<td>2015</td>
<td>926</td>
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<tr>
<td>6</td>
<td>Dulvy N K et al.</td>
<td>Extinction risk and conservation of the world's sharks and rays</td>
<td>EIlfe</td>
<td>2014</td>
<td>732</td>
</tr>
<tr>
<td>7</td>
<td>Edgar G J et al.</td>
<td>Global conservation outcomes depend on marine protected areas with five key features</td>
<td>Nature</td>
<td>2014</td>
<td>725</td>
</tr>
<tr>
<td>8</td>
<td>Chapron G et al.</td>
<td>Recovery of large carnivores in Europe's modern human-dominated landscapes</td>
<td>Science</td>
<td>2014</td>
<td>651</td>
</tr>
</tbody>
</table>

Smart agriculture: India
Table 4 — List of hot papers (green colour denoted the Indian papers and blue for global).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Authors</th>
<th>Article Title</th>
<th>Journal Name</th>
<th>Publication Year</th>
<th>Cited Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neutral Network</td>
<td>TRY plant trait database - enhanced coverage and open access</td>
<td>Global Change Biology</td>
<td>2020</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>Reid A J et al.</td>
<td>Emerging threats and persistent conservation challenges for freshwater biodiversity</td>
<td>Biological Reviews</td>
<td>2019</td>
<td>157</td>
</tr>
<tr>
<td>3</td>
<td>Bekun F V, Alola A A and Sarkodie S A</td>
<td>Toward a sustainable environment: Nexus between CO2 emissions, resource rent, renewable and nonrenewable energy in 16-EU countries</td>
<td>Science of the Total Environment</td>
<td>2019</td>
<td>148</td>
</tr>
<tr>
<td>4</td>
<td>Barlow J et al.</td>
<td>The future of hyperdiverse tropical ecosystems</td>
<td>Nature</td>
<td>2018</td>
<td>86</td>
</tr>
<tr>
<td>5</td>
<td>Meyfroid P et al.</td>
<td>Middle-range theories of land system change</td>
<td>Global Environmental Change-Human and Policy Dimensions</td>
<td>2018</td>
<td>62</td>
</tr>
<tr>
<td>6</td>
<td>Brancalion P H S et al.</td>
<td>Global restoration opportunities in tropical rainforest landscapes</td>
<td>Science Advances</td>
<td>2019</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>Chenu C et al.</td>
<td>Increasing organic stocks in agricultural soils: Knowledge gaps and potential innovations</td>
<td>Soil &amp; Tillage Research</td>
<td>2019</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>Diaz S et al.</td>
<td>Pervasive human-driven decline of life on Earth points to the need for transformative change</td>
<td>Science</td>
<td>2019</td>
<td>44</td>
</tr>
</tbody>
</table>
(Table 3 & 4) i.e. there is need of improvement in the research work in the field of smart agriculture in India.

Conclusion

Though India ranks ahead in total number of publications, it lags behind in highly cited papers. It indicates need for quality work to gather more citations and improve the country’s position in quality publishing. Academic stages like this may help in this regard. On growth trend of publications, India performs better with higher growth rate compared to world growth trend in almost all areas of our search here especially in the area of smart agriculture but for “biodiversity”, where it has to concentrate and come up with good number of quality publications to stay ahead. These observations are, though authentic, but incomplete without looking into other equally good sources viz., Scopus, Google Scholar.

References

Abstract

Global health diplomacy has given birth to vaccine diplomacy and later it got linked with vaccine science diplomacy which itself an amalgamation of science diplomacy. Since India is leader in vaccine manufacturing, it contributes approximately 60% of vaccines to the global vaccine supply. According to the present scenario, India is harnessing the power of soft skill by offering COVID-19 vaccines to its immediate neighbors to leverage diplomacy. Vaccine diplomacy could serve humanity and suffering countries if significant collaborations and efforts by global entities are to be done on the multilateral level.

Key words: Influenza, vaccine, pandemic, antipoverty vaccine, ascariasis and trichuriasis

Introduction

The world is going through a tough time and facing the worst situation ever due to the COVID-19 pandemic, but even in normal time, up to half of the world's tropical diseases are being ignored. Most commonly it includes diseases such as schistosomiasis, leishmaniasis, hookworm infection, leprosy, rabies, trachoma, dengue, lymphatic filariasis, etc; these can severely cause long-term disabling health effects. The adverse disabling can affect the rural working sectors, particularly in the developing countries where the lack of proper treatment directly affects the ailing persons and making them difficult to work that directly affects our economic productivity. Because these diseases mostly affects the poor sections of the society therefore the health conditions of girls and women as well as child development, the vaccines under development at Sabin are sometimes referred to as the antipoverty vaccines [1,2]. This ultimately can lead to various insecurities, including agricultural and industrial challenges. There is a likelihood of increased conflict among groups and states. Thus, the question on cheaper alternatives and preventive therapeutics like vaccines, the ability to resolve conflict, and how it can nurture diplomacy pops, continuously provokes. Some of the first-world countries including the United States and the United Kingdom have established scientific ties with the developing countries to implement potentially strong vaccine diplomacy.

The production of polio vaccine and its implementation is a tremendous example showing how vaccine diplomacy has driven collaboration and coordination in times of crisis, allowing nations to put aside their dogmatic differences to eradicate a pandemic. The way diplomatic efforts have been put in place to counter various other diseases such as cholera, measles, hepatitis, Ebola, etc. This is how a meaningful programme on vaccine research and development opens the door of uncounted opportunities to potentially improve and strengthen foreign relations and promote peace, thus empowering science diplomacy.

The miraculous term Vaccine was first used in 1798 when cowpox was administered as an inoculum to prevent smallpox. It is derived from Vacca, which in Latin is used for the cow. Since Smallpox was a massive killer epidemic, the first vaccine immediately got acclaim at the beginning of the 19th century. The next set of vaccines,
including a new rabies vaccine, was developed almost one hundred years later by France’s Louis Pasteur [3,4].

Vaccine diplomacy flourished in the later decades of the 20th century. According to WHO’s, Health as a Bridge to Peace-Humanitarian Cease-Fires Project (HCFP), vaccines and vaccinations were used to negotiate so-called “days of tranquillity” in more than a dozen countries during the 1980s and 1990s, including Afghanistan, Angola, Chechnya, Democratic Republic of Congo, El Salvador, Guinea Bissau, Iraq, Lebanon, Philippines, Sierra Leone, Sri Lanka & Sudan [5].

At the beginning of 2001, the broad frame work of global health diplomacy outlined above helped to generate the concepts of vaccine diplomacy and vaccine science diplomacy. Vaccine diplomacy refers to almost any aspect of global health diplomacy that relies on the use or delivery of vaccines and encompasses the important work of the GAVI Alliance, as well as elements of the WHO, Gates Foundation, and other important international organizations. Central to vaccine diplomacy is its potential as a humanitarian intervention and its proven role in mediating cessation of hostilities and even cease-fires during vaccination campaigns[5-7,8]. In such cases, an international organization, such as WHO or the United Nations Children’s Fund (UNICEF), or an associated nongovernmental organization may play a central role.

Science Diplomacy: The concept of science diplomacy itself is much debatable, considering its recent origin. It was not before the golden age of the 21st century that science diplomacy activities were becoming popular and attempted to practice by academicians, politicians, stakeholders, and activists. The concept of science diplomacy involves practices in numerous ways that broadly includes the practice of making international decisions involving science to fulfil a definite and common goal.

The definition of science diplomacy is still evolving and considering its relation among different sectors of international relations. It can therefore be considered as a subcategory of the arena of international relations that fosters the interaction among scientists, researchers, officials, politics, activists, research-educational institutions, diplomats, and stakeholders.

Science diplomacy is mainly classified into 3 major categories that include “Science in Diplomacy” where scientific knowledge is used to advise, inform, or support foreign policy objectives, “Science for diplomacy”, where scientific cooperation may improve international relationships, and “Diplomacy for science”, where diplomacy facilitates international scientific cooperation. These 3 categories are based on several actions that include the actions that intents to advance a country’s national needs, set opportunities to meet global challenges and needs, and designed to address international interests. The actions were framed by the present and former science advisers to the Foreign Ministers of the United States, the United Kingdom, New Zealand, and Japan.

Some of the examples of Science Diplomacy encompasses the bi-polar world (to avoid any detrimental use of technology during the cold war between the US and the then Soviet Union), non-proliferation treaty, climate conventions, 1-2-3 agreement providing access to nuclear fuel for civilian uses, the launch of satellites for other countries by select countries, working on energy security through International Solar Alliance, etc.

Indispensable Science Diplomacy: The soft power of science has the potential to reshape global diplomacy” (Ahmed Hassan Zewail). Science diplomacy is crucial for foreign affairs and address challenges shared with other nations. The global challenges include issues related to research funds, international research partnerships, global organization, future of public policy, and global governance. It is important to promote scientific cooperation, to fence any diplomatic failures, and to reduce the potential for conflict among countries or international scientific organizations.
Leveraging science diplomacy to increase cooperation and collaboration is a demand of the hour. Whenever we talk about fighting a crisis that is affecting most of the parts of the world, diplomacy always emerges as a major milestone not only in terms of collaboration but also from sharing resources without any extra cost which India has already done. As we know Science and Technology can’t work in a vacuum since multilateral collaborations and efforts are becoming the bridge to fulfill the gap of sustainability.

**Vaccine Diplomacy along with Vaccine Science Diplomacy:** The Right to adequate healthcare flows from the sanctity of human life and the dignity that belongs to all persons. Health is a fundamental right, which has as its prerequisites social justice and equality. It should be accessible to all. It is not simply the right not to be unwell, but to be well. It encompasses not just the absence of disease or infirmity, but “complete physical, mental and social well-being”, and includes freedoms such as the right to control one’s health and body and to be free from interference (for instance, from non-consensual medical treatment and experimentation), and entitlements such as the right to a system of healthcare that gives everyone an equal opportunity to enjoy the highest attainable level of health.

Vaccine diplomacy is one of the branches of global health diplomacy, which refers to both- a system of organization & means to communicate as well as negotiate processes that help to shape the sphere of health that provokes its determinants in the sector of the global policy environment. Vaccine diplomacy is predominant for the use and delivery of vaccines among different global locations. Being a hybrid of global health diplomacy and science diplomacy, vaccine diplomacy offers innovative and unique approaches for promoting foreign policies and diplomatic relations between nations, particularly between adversarial nations. Vaccine science diplomacy leads to the testing of vaccines of some highly neglected diseases and innovative opportunities to develop vaccines to combat diseases, especially the Neglected tropical diseases (NTDs) and other emerging novel diseases. It was eminent that many developing countries were on the “outside looking in” when it came to having access to influenza vaccines, including the vaccine for the H1N1 pandemic influenza in 2009 and prototype H5N1 avian influenza vaccines[9,10]. These types of activities acquire heavy scientific input, international cooperation, and compromise on issues between nations.

An underlying theme of both vaccine and vaccine science diplomacy is that vaccines are unique in comparison to other medical or public health interventions. By some estimates, vaccines are the single most powerful intervention ever developed by humankind in terms of the lives that they save. By one estimate, modern vaccines have saved more lives than those that were lost in the world wars during the 20th century. Vaccine Science Diplomacy is within the overall ambit of Vaccine Diplomacy with inputs both from Science Diplomacy and Global Health Diplomacy. Scientists are major contributors not only in the development of Vaccines but also in the related technologies. There are situations when scientists of more than one nation (even nations hostile to each other) work together to develop these techniques and produce a vaccine based on scientific interactions.

**The dominance of Vaccine Science Diplomacy:** Collaboration and partnership between nations and global organizations may lead to an extreme increase in the utilization of vaccine diplomacy and vaccine science diplomacy in foreign policy. Given the strong legacy and power of international scientists, vaccine developers, government officials, and global health practitioners joining their hands together for the same goal can improve health everywhere in the world. The improved smallpox vaccine which resulted in an international collaborative effort is a great example of the naturally occurring smallpox disease that was eradicated during the late 1970s. This effort shows the power of vaccine science diplomacy and
manifested the work of scientists across political and geographical barriers, and ultimately, helped overcome the pandemic.

Vaccine diplomacy is providing an excellent opportunity for almost every country to strengthen their weaknesses in the field of science diplomacy as well as improve their policy to nourish the environment of technology transfer. Though countries sometimes face personal dilemmas to share their resources with partner countries at last this will going to help its citizens so no harm in that. History of science diplomacy taught us that whenever the world is fighting for a common problem we must collaborate with solidarity on an international level without hampering our dogmas. The renowned French-biologist, Louis Pasteur once remarked, “Science knows no country because knowledge belongs to humanity”.

**Potential of Vaccine Science Diplomacy to restrain the COVID-19:** Nearly after a century of Spanish flu, humanity has faced another global threat: “The COVID-19 pandemic”. Countries should become the backbone to each other with which the globe can win the battle against this deadly virus in a more united way because the pandemic is global so that our strategies should be global and collaborative. To overcome this crisis, we need international collaboration, strong relationships between nations, and effective diplomacy. This is impossible without global ties among scientists of different nations, which have to be facilitated by the governments. Scientists, however, have long term formed relationships with their colleagues all across the world, even if their government hasn’t got along.

The COVID-19 pandemic is a golden opportunity for scientists and the governments to engage with the public and so internationally. As science and technology rely on transparency and international collaboration promoting these values can nurture the change we need to solve the problems of the present and the future.

Even as many nations are battling the deadly pandemic, India, a world leader in health, has lent a helping hand by exporting vaccines to them. During the pandemic, India also proved to the world that it has the best medical facility in comparison to most developed countries. India even reached out to supply Covid-19 medicines such as Hydroxychloroquine and Remdesivir, diagnostic kits, and equipment such as masks, gloves, and ventilators. Many of these supplies were given as gifts to partner nations. India also conducted virtual camps to train healthcare workers, especially on how to conduct tests.

Social media has also played a great role in aiding the process by making internationally collaborated webinars a trend. While we all know now, the terms most of us have never heard of before, like flattening the curve, quarantine, physical distancing, and herd immunity. Unconsciously, modern digital media in a sense is helping to bring countries together, keeping us informed and strengthening science diplomacy.

Recently, the government asked for 45 lakh more doses from the makers of Covaxin and even allowed the manufacturers to provide vaccines to other countries. The first batch of one lakh doses of Covishield was delivered to Bhutan and Maldives. Besides, Nepal and Bangladesh too have received the vaccines. All four got it under the “Neighbourhood First” policy. Many other countries are in line to receive the vaccine from India. The ministry of external affairs has said that India will supply the vaccines to partner countries over the coming weeks and months in a phased manner, keeping in view the domestic requirements. With this, India has proved that it is the leading country in the field of medicine. Besides cementing diplomatic ties with other countries, it will also help to increase medical tourism.

**Conclusion**

Science diplomacy has proved itself a successful milestone in developing vaccines for some deadly diseases, such as Polio, Tuberculosis, Smallpox, Tetanus, etc as well as emerged as a blessing for combating diseases, like SARS, Ebola, and Zika. It similarly continues

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to be extremely relevant during the COVID-19 pandemic today, through sharing of resources, research data, ideas, and rules and regulations.

Science is unquestionably facilitating diplomatic relations moreover allowing adversarial and conflicting nations to come together and find a definite solution to common global issues as quickly as possible. In a time of emergency, we must need Substantive joint collaborative efforts by local communities and global entities are required to solve global issues through science diplomacy. A proper overarching framework has not yet been formed to measure the expanded role of science diplomacy in foreign policy. Establishing such a framework might be useful in facilitating engagement between countries that have historically maintained tense for years.

No country is left behind by this deadly virus and we need to learn, design, and develop tools that can promote international relations particularly in the scientific arena to harness science diplomacy for the advancement & improvement of mankind.

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