Cloning, expression, purification of active recombinant human 12R-LOX enzyme and its inhibition by *Acalypha indica* extracts

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12R-LOX over-expression has been reported in various pathologies such as psoriasis, proliferative dermatitis as well as pulmonary obstructive diseases indicating that this enzyme plays significant role in pathogenesis of inflammatory diseases. 12R-LOX, therefore, is a suitable target for therapeutic intervention with potential application of its inhibitors in the treatment of skin and other inflammatory disorders. Identification of such inhibitors requires sufficient quantity of active enzyme to be produced by an easy and cost effective expression systems and further development of a robust assay system to screen inhibitors against the 12R-LOX enzyme. Therefore, in the present study, a prokaryotic expression system was developed to over-express and purify active human 12R-LOX enzyme by a single step purification process. We have further standardized an HPLC based assay system to assess the activity of purified human 12R-LOX enzyme. We show here that purified 12R-LOX preferentially utilizes free arachidonic acid as the substrate but it is also active on methyl ester of arachidonic acid, albeit less efficiently. Additionally, using this assay system we observed the potent inhibition of human 12R-LOX enzyme activity by the ethyl acetate and aqueous sub-fractions of *Acalypha indica* leaves, which is widely used in traditional medicines for the treatment of various skin ailments

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Abstract

12R-LOX over-expression has been reported in various pathologies such as psoriasis, proliferative dermatitis as well as pulmonary obstructive diseases indicating that this enzyme plays significant role in pathogenesis of inflammatory diseases. 12R-LOX, therefore, is a suitable target for therapeutic intervention with potential application of its inhibitors in the treatment of skin and other inflammatory disorders. Identification of such inhibitors requires sufficient quantity of active enzyme to be produced by an easy and cost effective expression systems and further development of a robust assay system to screen inhibitors against the 12R-LOX enzyme. Therefore, in the present study, a prokaryotic expression system was developed to over-express and purify active human 12R-LOX enzyme by a single step purification process. We have further standardized an HPLC based assay system to assess the activity of purified human 12R-LOX enzyme. We show here that purified 12R-LOX preferentially utilizes free arachidonic acid as the substrate but it is also active on methyl ester of arachidonic acid, albeit less efficiently. Additionally, using this assay system we observed the potent inhibition of human 12R-LOX enzyme activity by the ethyl acetate and aqueous sub-fractions of *Acalypha indica* leaves, which is widely used in traditional medicines for the treatment of various skin ailments.

Introduction

Lipoxygenases (LOXs) is a class of nonheme iron oxygenases that catalyze the conversion of 20-carbon polyunsaturated fatty acid - arachidonic acid (AA) and other polyunsaturated fatty acids to their hydroperoxy derivatives [1,2], which are further, metabolized to various bioactive lipid mediators such as leukotrienes, lipoxins, hydroxyeicosatetraenoic acids (HETEs), and

hepoxilins. These products are involved in myriad of biological events like immunity, inflammation, cell differentiation and proliferation [3,4,5]. Lipoxygenases stereo- and regioselectively oxygenate the substrate, arachidonic acid, and are thus named as 5-, 8-, 12- and 15-LOX [6]. 12R-Lipoxygenase (12R-LOX), is unique in incorporation of molecular oxygen on substrate arachidonic acid, R-stereospecifically, to produce 12R-Hydroperoxyeicosatetraencoic acid (12R-HPETE), which is acted upon by another epidermal lipoxygenase , eLOX-3, to generate hepoxilins, the potent signaling molecules [7,8].

12R-LOX has drawn much attention due to its inductive role in oxidising complex lipids thereby modifying membrane structure, which is critical for maturation, differentiation, induction of inflammatory pathways as well as proliferative potential of the skin cells [9,10,11,12]. Pivotal role of 12R-LOX in skin physiology came into light with the discovery of high levels of 12R-HETE in psoriatic skin cells and also in various carcinoma cell lines [13,14,15]. Additionally, high 12R-LOX activity has also been involved in pathogenesis of pulmonary obstructive diseases where it has been established that 12R-LOX stimulates mucin production by ERK activation and sp1 translocation [16]. From the above studies it appears that a critical amount of 12R-LOX enzyme activity is essential for maintaining cell homeostasis and overexpression of the same leads to pathological conditions. Thus, 12R-LOX represents a suitable target for therapeutic intervention where 12R-LOX inhibitors will be potential agents of therapeutic value in such pathologies.

Although cell extracts have been widely used as an enzyme source for biochemical assays of various LOX enzymes but they have limitations in terms of protein abundance, stability and less specific activities for the respective enzymes. The presence of the reducing factors, redox environment and endogenous inhibitors in the cells affect the catalytic activity of enzymes

when cell lysate were used as the enzyme source [17,18,19]. Although, there are reports that recombinant active 12RLOX enzyme was produced using mammalian cell lines yet it showed low catalytic activity [20,21,22]. For structural studies, human 12R-LOX (h-12R-LOX) enzyme was purified in soluble fraction only when it was co-expressed with bacterial chaperones, GroES and GroEL, in an *Escherichia coli* expression system [23]. Thus, there is a need of a system, which can be easily used to produce sufficient quantity of active h-12R-LOX enzyme, for its biochemical studies.

Traditionally medicinal plants have been used extensively to treat inflammatory disorders. Using the ethno-medicinal knowledge, from the traditional system, scientific studies are being undertaken to validate the potential of these plants, which provide insight into the scientific basis of natural remedies being practiced for centuries. *Acalypha indica* (Euphorbiaceae), commonly known as Indian nettle, is an annual herb, which grows in warm climate. It has been used to treat skin disorders in Asian countries [24,25,26]. Anti-inflammatory and anti-cancerous properties have been attributed to the extracts of this plant [27,28,29].

In the present study we report here the cloning and expression of His-tagged human-12R-LOX protein in BL21-Rosetta strain. We have purified this enzyme by Ni-NTA affinity chromatography and subsequently RP-HPLC based assay system was employed to assay the activity of the enzyme. Activity assay studies reveal that h-12R-LOX enzyme preferentially utilizes arachidonic acid as its substrate and methyl ester of arachidonic acid also, but with less specificity. Nevertheless, we have identified that the sub-fractions of *Acalypha indica* leaves significantly inhibit h-12R-LOX enzyme activity.

Materials & Methods

The chemicals used were as follows: Arachidonic acid, Arachidonic acid methyl ester from Cayman chemical Co. Inc. ; isoproplyl β-D- thiogalactopyranoside from Sigma ; sodium borohydride , ampicillin from Invitrogen and HPLC solvents from Merck ; Restriction enzymes were from New England Biolabs Inc.., Polyclonal h-12R-LOX Antibody, Sigma (HPA024002).

Cloning and Expression of human *12R-LOX* gene

Human *12R-LOX* gene was cloned and expressed as an N-terminal His-tagged fusion protein in bacterial expression vector pET28(b) , in order to express and purify the protein from bacterial host. Coding region of the cDNA of human *12R-LOX* was amplified by polymerase chain reaction from the mammalian cell expression vector pCMV6Neo-12*R*-LOX (OriGene, USA) using forward and reverse primers having NdeI and HindIII restriction sites, respectively (**F**-5′ GCAGCCCATATGGCCACCTACAAAGTCAGG 3′, **R**-5′ GCGCTCAAGCTTCTAAATAGAAATGCTGTTCTC 3′). The amplified fragment was digested with NdeI and HindIII and cloned into pET28(b), a prokaryotic expression vector. BL21(DE3)-Rossetta, an engineered strain of BL21, was then used to transform the pET28 (b) recombinant vector containing Kanamycin and chloramphenicol resistance gene as the selection markers. Single colony selected on kanamycin and chloramphenicol was checked for the expression of the protein. For this, single colony was cultured at 37^{o}C in LB medium containing 50mg/ml kanamycin and 25mg/ml chloramphenicol to reach an absorbance of 0.6 at 600nm. The cultured bacteria were then induced to express recombinant protein by using isopropyl β-D-thiogalactopyranoside (IPTG) (1mM final concentration) at 18^{o}C. The culture was incubated for 8-12 hrs and then the culture was spun down to collect the bacterial cells. The cells were

resuspended in the required amount of phosphate buffer saline (PBS) containing protease inhibitor. The cells were then lysed by sonication to extract protein. The cells were sonicated for 5 min. and then debris was removed by centrifugation at 13000 rpm for 30 min at 4°C. The clear supernatant was collected and was used as the source of enzyme.

Purification of the recombinant fusion protein

Purification of the recombinant protein was performed using nickle-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography as per manufacturer's protocol (Qiagen). Briefly, Ni-NTA slurry (500 microltrs for the extracts of 150 ml of culture) added to the cleared bacterial lysate and mixed gently by shaking at 4°C for 60min. Then the lysate – Ni-NTA slurry was loaded into a column and washed twice with wash buffer containing 20mM imidazole and 300mM NaCl. After washing the column to remove non-specific proteins, the 12R-LOX protein was eluted with buffer containing 250mM imidazole, which displaces the His-tag fusion protein from the matrix. The purified protein was fractionated and the fractions were analysed further by SDS-PAGE and confirmed by western blotting analysis and MALDI. The polyclonal anti12R-LOX antibody from Sigma (HPA024002), used for western blotting.

12R-LOX enzyme activity assay

Either purified protein or bacterial cell lysate which expressed 12R-LOX protein was added to a total volume of 0.5 ml of 1X phosphate buffered saline (pH 7.4). The substrate concentration used was 0.1 mM (final concentration) Arachidonic acid (free acid) or Methyl ester. The mixture was vortexed and incubated for 15 min at 37° C. After the incubation time, hydroperoxy fatty acids formed in the reaction were reduced to their more stable hydroxy derivatives by addition of sodium borohydride. After 5 min the reaction was stopped by acidification to pH 3 using 50 µl of acetic acid and then the proteins were precipitated with the addition of 0.5 ml of ice cold

methanol. The total mixture was then centrifuged (14000 rpm for 15 min) to remove the proteins and the clear supernatant was analysed on Reverse phase HPLC (RP-HPLC). High-pressure liquid chromatography was performed on a Shimadzu system equipped with Hewlett-Packard diode array detector 1040A and HP-Chemstation program. The solvent system used was methanol: water: acetic acid in the ratio of 85: 15: 0.1 with a flow rate of 1 ml/min. The chromatograms were followed by monitoring the absorbance for 12*R*-HETE at 235nm.

Source of *Acalypha indica*

The leaves of *Acalypha indica* were collected from Japali Teertham, Tirumala Hills of Andhra Pradesh, South India in January 2012 and identified by Dr. K.Madhava Chetty, Plant Taxonomist, Sri Venkateswara University. Sample collection area is located at height of 872 MSL, latitude of 13º42'2.6280N" and longitude of 79º20'21.0048E". No specific permission was required to collect the plant sample, as *Acalypha indica* is not an endangered or protected species. A voucher specimen has been deposited in the herbarium of the Department of Botany, Sri Venkateswara University, Tirupati. After collection these leaves were washed and sun-dried for 5-7 days. Coarse powder of these leaves was prepared in grinding machine.

Preparation of different solvent extracts of *Acalypha indica*

Grounded powder of the leaves of *Acalypha indica* weighing 250g was tightly packed to the 1/3 volume of the soxhlet apparatus and solvents with different polarity were used to extract the products. Methanol, ethanol, chloroform, hexane, ethyl acetate and water were used as the solvents for extraction. The extracted fractions were dried to remove the respective solvents. Each fraction was then dissolved in DMSO solvent. These fractions with different chemical

constituents were then used to check the inhibitory activity against h-12R-LOX, as per the HPLC based assay described above.

Results

Expression of h-12R-Lipoxygenase in prokaryotic expression host.

pCMV6 vector harbouring *h-12RLOX gene* (with 5′ and 3′ UTR) was obtained commercially from OriGene, USA. The gene was sequenced and its expression was checked by immunofluorescence after transient transformation into HEK cell line (Fig. S1). To express the gene in bacteria, ORF of *h-12R-LOX* gene was amplified using suitable primers and cloned into pET28b vector to generate pET-12R-LOX (12R-LOX in-frame with 6X His tag), as described in Materials and Methods. pET-12RLOX vector was initially transformed into BL21-pLys strain and induced with IPTG to check protein expression. We could not see induction in SDS-PAGE analysis (Fig. 1B), however, we could detect low amount of protein in western blot performed with anti-12R-LOX antibody (Fig. 1D). Analysis of nucleotide sequence of *h-12R-LOX* gene revealed presence of codon for few amino acids (Proline, Arginine etc.) which are rare in *E. coli* genome (Fig. 1F) and thus tRNA for these codons are depleted in *E. coli* cells. Hence, codon bias was suspected for the low protein production in the BL21-pLys strain. To circumvent this problem, pET-12R-LOX vector was transformed into BL21-Rosetta strain; which is engineered to have tRNA for these unique codons, and indeed protein of expected size (size~80 kDa) was expressed in considerable amount upon induction with 1 mM IPTG (Fig. 1A and B). The identity of protein was confirmed by western blot analysis using anti-12R-LOX antibody (Fig. 1C) and anti-His antibody (Fig. 1E) The over-expressed protein band was further subjected to MALDI analysis to reconfirm human 12R -LOX protein's identity (Fig. S3).

Purification of active recombinant His-tagged h12R-LOX.

Recombinant His-tagged h-12R-LOX enzyme was over-expressed and purified using Ni-NTA affinity chromatography as described under the Materials and Methods. The protein was purified and analysed on SDS-PAGE (Fig. 2A). We tried to further purify the protein using Gel filtration column but we couldn't separate it from two other smaller proteins (Fig. S2). Over-expressed proteins tend to associate with the GroEL and other chaperones and hence at this stage we speculate that these co-purified proteins which we could see in addition to our protein of interest could possibly be chaperones.

Purified h-12RLOX was confirmed by western hybridization (Fig. 2B). Elution fragments E_2 and E_3 contained large amount of proteins as was observed in SDS-PAGE. Flow through (unbound proteins) also had large amount of 12R-LOX protein which is possible in case of all Ni-NTA beads due to saturation with the proteins. As increased Ni-NTA beads will only increase the non-specific protein binding, in this study we used the amount of Ni-NTA beads where we got maximum purity of proteins.

Standardization of activity assay for purified h-12R LOX enzyme

Though, different groups have expressed 12R-LOX protein in prokaryotic and eukaryotic expression vectors but have achieved minimal enzymatic activity [1,21,23,32]. Therefore, we decided to first check the activity of the purified 12R-LOX enzyme on its known substrate. Reverse-phase HPLC based assay was standardized to check the activity of h-12R-LOX enzyme. The assay conditions and steps were used as described under Materials and Methods.

We started with characterizing the enzyme for its activity using IPTG induced BL21-Rosetta cell extracts expressing h-12R-LOX enzyme (Fig. 3). For all these assays 0.1 mM arachidonic acid or its methyl ester was incubated with protein concentrations of 3 mg/ml of cell extracts. In all the chromatograms we see a common peak (X), which is seen also in the negative

control for the experiment (Fig. 3B). Commercially bought 12*R*-HETE (Cayman Chemical Company, Ann Arbor, MI, USA) was injected and run on RP-HPLC for the standard (Fig. 3A). Peak 'Y' denotes 12*R*-HETE product. RP-HPLC analysis of the incubation of the cell extracts with free acid form of arachidonic acid showed the formation of 12*R*-HETE peak as only product with ~300 mAU at 235 nm (Fig. 3D- product specific peak is marked as Y). We didn't observe any 12*R*-HETE product peak when methyl ester of arachidonic acid was used as the substrate (Fig. 3C). Chromatograms for both negative control and experimental when methyl ester of arachidonic acid (AA) as the substrate showed similar patterns (Compare Fig. 3B and Fig. 3C).

Then purified enzyme was used to do RP-HPLC assay with both the substrates. (Fig. 4). 200 µg/ml of purified h-12R-LOX enzyme was incubated with 0.1 mM substrates and products were analysed on RP-HPLC. 12*R*-HETE product peak 'Y' (~350-400 mAU) was observed when free arachidonic acid (AA) was used as the substrate (Fig. 4C). Notably, we could also see a product peak when methyl arachidonate was used as a substrate though absorbance of the peak was limited to only~50 mAU (Fig. 4B). While using purified enzyme as the enzyme source for our assays, we saw two common peaks 'X' and 'Z' in all the chromatograms including negative control (without substrate) (Fig.4A). NDGA (nordihydroguaiaretic acid), which is a standard LOX inhibitor was used in order to confirm the nature of the peak. The addition of NDGA inhibited the formation of 12*R*-HETE product confirming that the peak 'Y' which appears is indeed a lipoxygenase product peak (Fig. 4D).

Extracts of *Acalypha india* leaves significantly inhibit h-12R LOX activity

For performing the inhibition assays we used purified enzyme and free arachidonic acid as a substrate. Inhibition assay was performed either in presence of DMSO as a positive control or in presence of different extracts of *Acalypha indica* dissolved in DMSO. In all, seven extracts of

231 *Acalypha indica* plant leaves were prepared with different solvents using soxhalation method and
232 tested for inhibition using RP-HPLC. Ethyl acetate and aqueous extracts of *Acalypha indica*
233 showed inhibition of 12R-Lipoxygenase enzyme activity up to 60-70%. Additionally we found
234 12R-LOX inhibition by Zileuton, which is widely used 5-LOX inhibitor (IC50- 0.7-6
235 micromolar) [31]. However, Zileuton inhibits 12R-LOX at a higher concentration (IC50; 210
236 micromolar). The inhibition of 12R-LOX by *Acalypha* extracts was significant and was
237 comparable to inhibition of 12R-LOX enzyme by zileuton (Fig.5). Here, it is important to
238 mention that the molecule responsible for 12R-LOX inhibition would be in very low
239 concentration in whole *Acalypha* extracts.

240 **Discussion**

241 12R-LOX is a lipoxygenase enzyme that utilizes polyunsaturated fatty acids such as
242 arachidonic acid, linoleic acid etc. to produce hydroperoxy products which further give rise to
243 variety of lipid mediators performing important cellular functions [3]. Considerably enhanced
244 activity of 12R-LOX enzyme is a common feature in psoriatic scales, proliferative dermatosis
245 and pulmonary obstructive diseases suggesting its pivotal role in such pathology [13,14,15,16].

246 Considering the importance of 12R-LOX enzyme, present study was undertaken to
247 produce sufficient quantities of lipoxygenase enzyme in cost effective and less time consuming
248 manner, so that the active enzyme can be used for various purposes such as biochemical studies,
249 identification of its inhibitors from natural sources, crystallization studies etc. Human 12R-
250 lipoxygenase was identified from human keratinocytes. Although it was cloned and expressed in
251 Hela cells but the enzyme showed very low catalytic activity [1]. Subsequently, Sun and co-
252 workers cloned both murine and human 12R-LOX and expressed in HEK293 and COS-M6 cells
253 but were unable to detect any enzyme activity [32]. Smita and co-workers, observed endogenous

overexpression of 12R-LOX in an epidermoid carcinoma cell line A431 [15]. We used the cell lysate of A431 cell line as the enzyme source for human 12R-LOX, but were unable to detect any enzymatic activity using HPLC assay system. Redox conditions or reducing factors or inhibitory factors present in whole cell lysate could be possibly inhibiting the enzyme activity. Other reports show that baculovirus system can be used to produce active 12R-LOX enzyme, which can be used for biochemical studies, but this method of enzyme production is laborious, costly and labor intensive. Meruvu and co-workers cloned and expressed murine 12R-LOX in *E. coli* and observed that expression of protein was very low [21]. Recently, it has been reported that purification of human 12R-LOX was achieved from bacterial host only when two chaperons have been co-expressed with h12R-LOX [23]. We report here the cloning, expression and purification of his-tagged human12R-LOX in BL21(DE3)-Rosetta strain, which takes care of codon bias. We have successfuly expressed and purified high amount of human 12R-LOX protein (12mg of purified protein from 1Ltr of Bacterial culture). The expression was relatively very high as compared to above mentioned reports, where people have tried expression of human 12-LOX protein in both prokaryotic as well as eukaryotic expression systems. [1,21,32]. Notably, we were able to produce this sufficient amount of protein without co-expressing chaperons as was done by Deb and co-workers [23]. Our assay system confirms that the overexpressed recombinant protein was fully active which suggests that expressed proteins are in their proper conformations.

Biochemical characterization of both human and mouse 12R-LOX enzymes has been done previously by different groups. Boeglin and co-workers have shown that the human12R-LOX enzyme, purified from baculovirus, utilised arachidonic acid as the substrate but with low catalytic activity [7]. They couldn't see any activity with methyl ester of AA. Siebert and co-

277 workers have shown that mouse 12R-LOX metabolizes methyl ester of arachidonic acid as the
278 substrate and does not utilize free AA [20]. Our results are consistent with earlier observations
279 as we found that free AA is the preferred substrate of human 12R-LOX enzyme in our assay
280 conditions. In addition to that we could also see week activity of human 12R-LOX enzyme on
281 methyl ester of AA, which has never been reported earlier. In earlier studies, the amount of
282 enzyme expression was limiting and since we have found out that human 12R-LOX has a very
283 low activity with methyl ester of AA, we suspect the limitation of enzyme was the reason in
284 earlier studies for not getting any activity when methyl ester of AA was used as a substrate.

285 Additionally, using this assay system we have found out inhibitory activity of 12R-LOX
286 enzyme by the fractions of *Acalypha indica*. *Acalypha indica* is being widely used in traditional
287 medication in various parts of the world, including India, Bangladesh, Sri Lanka, Africa and the
288 Philippines. Apart from possessing the properties like diuretic, purgative and anthelminthic, it is
289 being used as a cure for bronchitis, asthma, pneumonia, scabies and other cutaneous diseases
290 [24,30]. Recent studies have attributed anti-inflammatory and wound healing properties to the
291 extracts of this plant [25,26, 27, 28, 29]. Enhanced activity of 12R-LOX has been hypothesized
292 as the driving factor towards the inflammatory diseased conditions [14,16,33]. So it was very
293 interesting to check if the anti-inflammatory property observed in *Acalypha indica* extracts is
294 related to inhibition of 12R-Lipoxygenase enzyme. We were able to see inhibition of human
295 12R-LOX enzyme activity by the aqueous and ethyl acetate extracts of plant *Acalypha indica*.
296 Perera and co-workers (2016) have recently reported the phytochemical analysis of *Acalypha*
297 *indica* , which reveals the presence of tannins, saponins, alkaloids, terpenes, steroids, flavanoids,
298 and reducing sugars in *Acalypha indica* extracts. Interestingly, a class of constituent compounds
299 known as saponins is seen to be commonly present in both the aqueous and ethyl acetate soxhlet

extracts of *Acalypha indica* leaves [34]. Notably, in our study we were also able to see the potent inhibition of 12R-LOX enzyme by these two soxhlet fractions- aqueous and ethyl acetate. So, these two observations strongly indicate that saponins could be the probable constituent compounds present in *Acalypha indica*, inhibiting the 12R-LOX enzyme activity. This hypothesis finds supportive evidence from the previous studies where specific types of saponins (Glycyrrhizin) and its derivatives (Glcyrrhetic acid) have been observed to inhibit lipoxygenases [35,36]. Thus, it would be an important study to identify the specific saponins present in *Acalypha* extracts responsible for inhibiting 12R-LOX enzyme activity.

The present study provides significantly improved protein expression system for recombinant 12R-LOX, which will prove useful for biochemical studies of 12R-LOX. HPLC based assay system established in this study provides a cell-free screening system for testing various plant based or chemically synthesized inhibitory compounds against 12R-LOX, in an accurate and highly reproducible manner. Our study, for the first time, reports the potential of *Acalypha indica* extracts to inhibit human12R-LOX related to various inflammatory diseases. These findings suggest that there is a possibility for *Acalypha indica* as a potential source for isolating a novel candidate molecule(s) for the treatment of inflammatory skin ailments.

Conclusions

In summary, we have successfully expressed and purified fully active, human 12R-LOX enzyme in native conditions, using a bacterial expression system. It is evident from our results that human-12R-LOX preferentially utilizes arachidonic acid and also exhibits very low catalytic activity towards arachidonic acid methyl ester. Importantly, using cell-free HPLC based assay

system we showed that aqueous and ethyl acetate extracts of *Acalypha indica* plant can potentially inhibit human 12R-Lipoxygenase activity. However narrowing down to the active ingredients and then validation of their anti-inflammatory effects by using cell-based test systems would help in advancing towards conclusive analysis of the therapeutic of *Acalypha indica*. Nevertheless the present study, in part could provide scientific basis to the therapeutic use of the plant in the traditional medicine for various inflammatory ailments.

Acknowledgements

Conceived and designed the experiments: NF and PR. Performed the experiments: NF and BPR. Analyzed the data: NF, NK and PR. Contributed reagents/materials: PR. Wrote the paper: NF and PR.

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Legends to the Figures

Fig. 1. Expression analysis of human 12R-LOX protein in *E. coli*. h-12R-LOX protein expression was analysed in BL21-Rosetta strain and BL21 (pLys) strain by the SDS-PAGE technique. (**A**) The SDS-PAGE (8%) gel shows expression profile of h-12R-LOX protein after 1 mM IPTG induction for 8 hrs and 16 hrs in BL21-Rosetta strain. un-uninduced, LMW-Low molecular marker (Amersham Biosciences). (**B**) Comparison of protein expression in BL21 (pLys) and BL21-Rosetta strain after IPTG induction. Lane 1 and 2 represent induced and uninduced samples from BL21 (pLys) strain respectively. Lane 3 and 4 represent induced and uninduced samples from BL21-Rosetta strain respectively. (**C**) and (**D**) Western blot analysis of 12R-LOX protein in BL21-Rosetta and in BL21 (pLys) strain respectively. Immunoblots were probed with anti-12R-LOX polyclonal antibody (Sigma), and detected by HRP conjugated secondary antibody. (**E**) Western blot analysis of 12R-LOX protein in BL21-Rosetta at 8hrs after induction, probed with anti-His antibody. **(F)** Codon Bias in *E.coli.* BL21-Rosetta strain has been engineered to express the tRNA's for these rare codons.

Fig. 2. Purification of recombinant h-12R-LOX protein by Ni-NTA agarose column chromatography. (A) 12% SDS-PAGE analysis of protein fractions of h-12R-LOX protein

following Ni-NTA agarose chromatography. FT, flow through collected after cell lysate passed through Ni-NTA agarose column; W, Wash fraction collected after washing the Ni-NTA agarose column; M, Protein molecular weight marker (Broad) (TAKARA BIO INC.) ; E_1, E_2, E_3 E_4 and E_5 are the eluted fractions of 6X His-tagged h-12R-LOX associated protein. **(B)** Western analysis of protein fractions of h-12R-LOX protein following Ni-NTA agarose chromatography. Immunoblots were probed with anti-12R-LOX polyclonal antibody (Sigma) and detected by HRP conjugated secondary antibody.

Fig. 3. HPLC analysis of the reaction products formed by BL21-Rosetta cell lysate expressing human-12R-LOX. (A) Chromatogram analysis for standard 12R-HETE **(B)** Chromatogram analysis when uninduced cell lysate was used as the enzyme source and incubated with Arachidonic acid (AA). **(C)** Chromatogram analysis when IPTG induced cell lysate was used as an enzyme source and incubated with substrate Arachidonic acid-methyl ester **(D)** Chromatogram analysis when IPTG induced cell lysate was used as an enzyme source and incubated with arachidonic acid (AA) substrate. All assays were done as described in materials and methods. Note that peak labeled as X is present in all the chromatograms including control (Fig. 3B). Peak Y was observed with standard 12*R*-HETE (Fig. 3A) and when arachidonic acid was incubated with induced cell lysate (Fig. 3D).

Fig. 4. HPLC analysis of the reaction products formed from substrate by purified human 12R-LOX. (**A**) Chromatogram analysis when purified human 12R-LOX was either incubated with DMSO control (without substrate), (**B**) or with methyl ester of arachidonic acid (**C**) or with arachidonic acid. (**D**) In another experiment, purified human 12R-LOX enzyme was first incubated with NDGA (general LOX inhibitor) and then substrate (arachidonic acid) was added to the reaction mixture. Note that peak labeled as X and Z are present in all the chromatograms

including control (Fig. 4A) where no substrate was added. Peak Y observed when Arachidonic acid was used as a substrate and peak Y disappeared when enzyme was preincubated with NDGA. All assays were done as described in materials and methods.

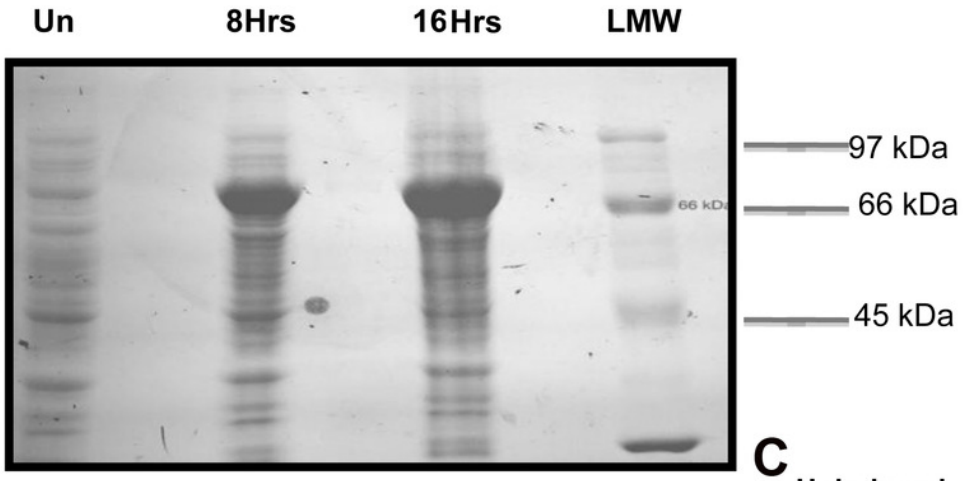
Fig. 5. HPLC analysis of the reaction products formed by purified human 12R-LOX in the presence of extracts of *Acalypha indica* leaves. Purified human 12R-LOX activity assay was assessed by incubating it with substrate arachidonic acid. In different experiments, the enzyme was pretreated with control DMSO (**A**), Zileuton (**B**), Ethyl acetate extracts of plant *Acalypha indica* (**C**), Aqueous extracts of plant *Acalypha indica* (**D**). Area of the peak was measured and inhibition of 12R-LOX enzyme activity was plotted quantitatively (**E**). The values are the average of 3 experiments only.

Figure 1

Expression analysis of human 12R-LOX protein in *E. coli*

h-12R-LOX protein expression was analysed in BL21-Rosetta strain and BL21 (pLys) strain by the SDS-PAGE technique. (**A**) The SDS-PAGE (8%) gel shows expression profile of h-12R-LOX protein after 1 mM IPTG induction for 8 hrs and 16 hrs in BL21-Rosetta strain. un-uninduced, LMW-Low molecular marker (Amersham Biosciences). (**B**) Comparison of protein expression in BL21 (pLys) and BL21-Rosetta strain after IPTG induction. Lane 1 and 2 represent induced and uninduced samples from BL21 (pLys) strain respectively. Lane 3 and 4 represent induced and uninduced samples from BL21-Rosetta strain respectively. (**C**) and (**D**) Western blot analysis of 12R-LOX protein in BL21-Rosetta and in BL21 (pLys) strain respectively. Immunoblots were probed with anti-12R-LOX polyclonal antibody (Sigma), and detected by HRP conjugated secondary antibody. (**E**) Western blot analysis of 12R-LOX protein in BL21-Rosetta at 8hrs after induction, probed with anti-His antibody. **(F)** Codon Bias in *E.coli.* BL21-Rosetta strain has been engineered to express the tRNA's for these rare codons.

A



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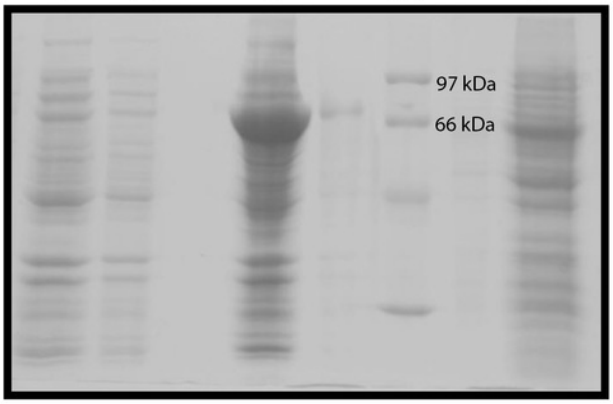

C

D

E

F

Amino acids	Major codon in *E. coli*	Major codon in h-12R LOX
Glycine	GGG/GGU/GGC	GGA/GGG
Proline	CCG/CCA/CCU	CCC

Figure 2

Purification of recombinant h-12R-LOX protein by Ni-NTA agarose column chromatography.

(A) 12% SDS-PAGE analysis of protein fractions of h-12R-LOX protein following Ni-NTA agarose chromatography. FT, flow through collected after cell lysate passed through Ni-NTA agarose column; W, Wash fraction collected after washing the Ni-NTA agarose column; M, Protein molecular weight marker (Broad) (TAKARA BIO INC.) ; E_1, E_2, E_3 E_4 and E_5 are the eluted fractions of 6X His-tagged h-12R-LOX associated protein. **(B)** Western analysis of protein fractions of h-12R-LOX protein following Ni-NTA agarose chromatography. Immunoblots were probed with anti-12R-LOX polyclonal antibody (Sigma) and detected by HRP conjugated secondary antibody.

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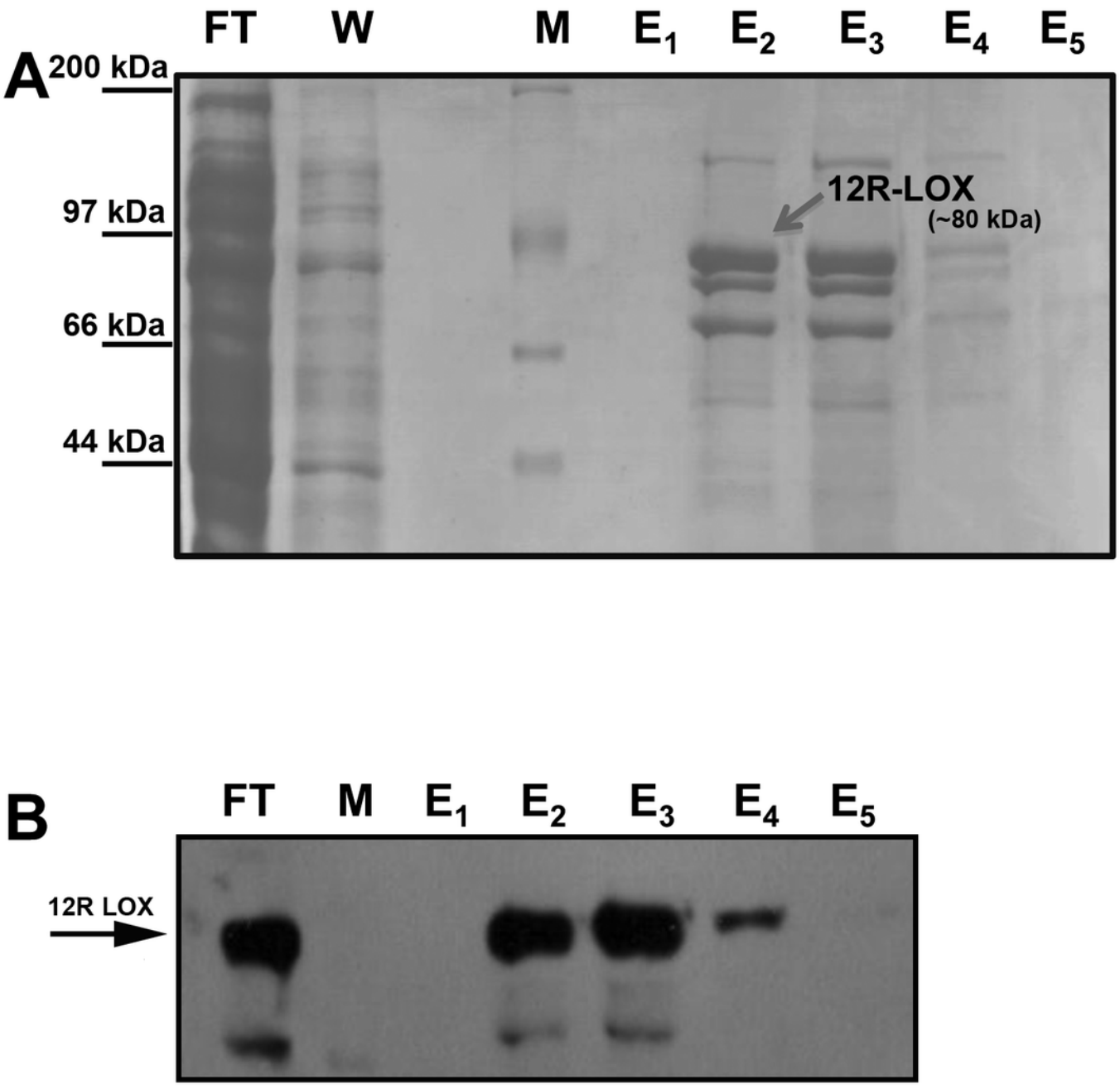

Figure 3

HPLC analysis of the reaction products formed by BL21-Rosetta cell lysate expressing human-12R-LOX.

(A) Chromatogram analysis for standard 12R-HETE **(B)** Chromatogram analysis when uninduced cell lysate was used as the enzyme source and incubated with Arachidonic acid (AA). **(C)** Chromatogram analysis when IPTG induced cell lysate was used as an enzyme source and incubated with substrate Arachidonic acid-methyl ester **(D)** Chromatogram analysis when IPTG induced cell lysate was used as an enzyme source and incubated with arachidonic acid (AA) substrate. All assays were done as described in materials and methods. Note that peak labeled as X is present in all the chromatograms including control (Fig. 3B). Peak Y was observed with standard 12*R*-HETE (Fig. 3A) and when arachidonic acid was incubated with induced cell lysate (Fig. 3D).

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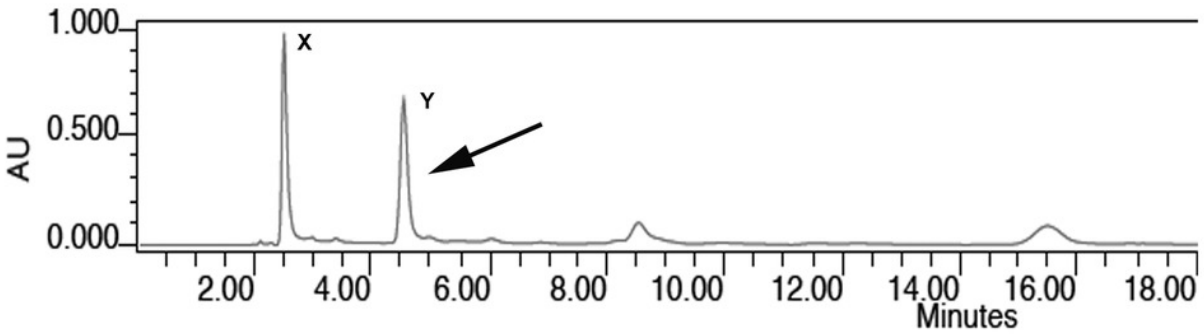
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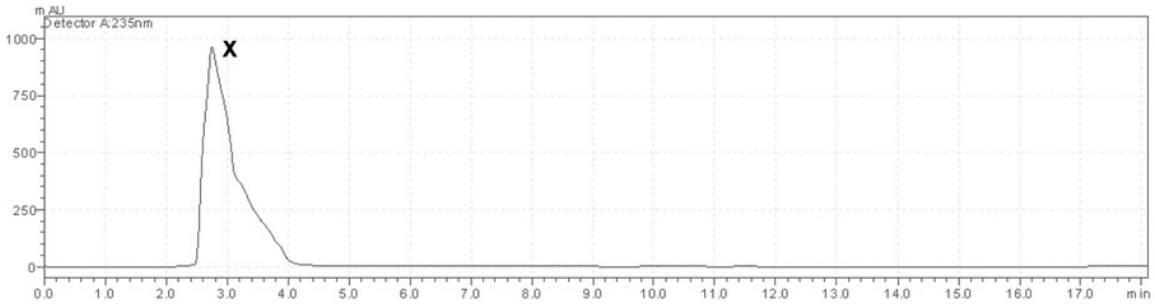
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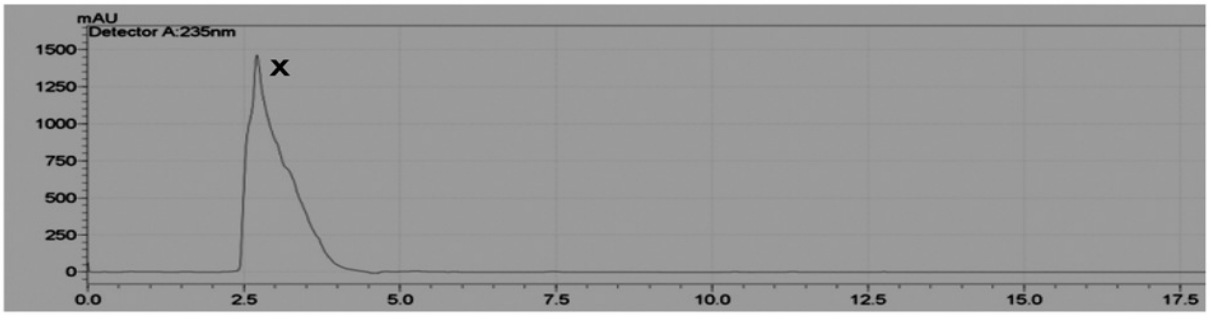
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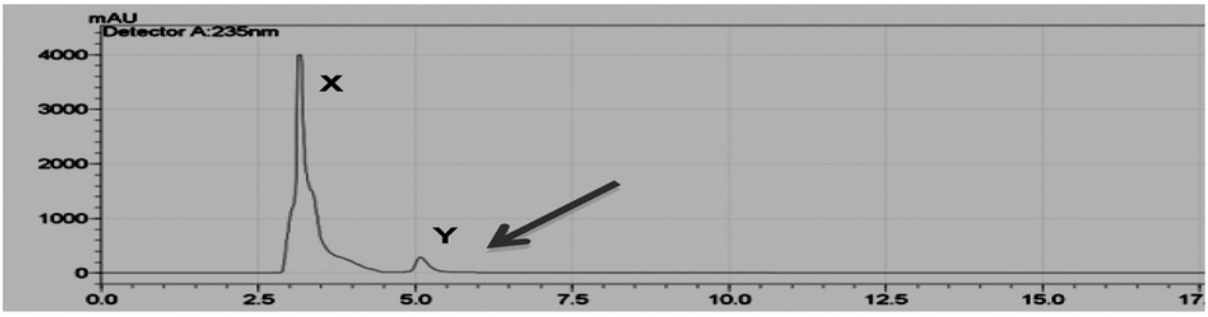
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Figure 4

HPLC analysis of the reaction products formed from substrate by purified human 12R-LOX.

(**A**) Chromatogram analysis when purified human 12R-LOX was either incubated with DMSO control (without substrate), (**B**) or with methyl ester of arachidonic acid (**C**) or with arachidonic acid. (**D**) In another experiment, purified human 12R-LOX enzyme was first incubated with NDGA (general LOX inhibitor) and then substrate (arachidonic acid) was added to the reaction mixture. Note that peak labeled as X and Z are present in all the chromatograms including control (Fig. 4A) where no substrate was added. Peak Y observed when Arachidonic acid was used as a substrate and peak Y disappeared when enzyme was preincubated with NDGA. All assays were done as described in materials and methods.

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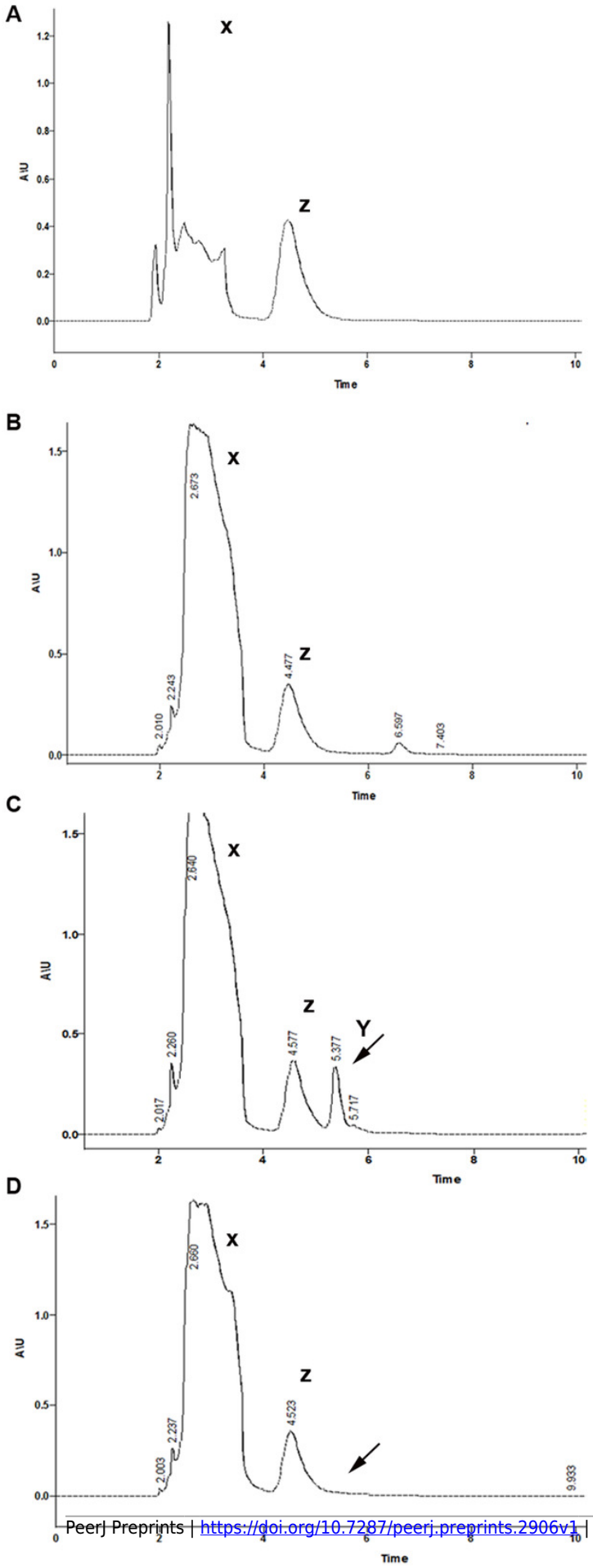
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Figure 5

HPLC analysis of the reaction products formed by purified human 12R-LOX in the presence of extracts of *Acalypha indica* leaves. Purified human 12R-LOX activity assay was assessed by incubating it with substrate arachidonic acid. In different ex

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