

High benzoic acid production in a grape-based Ayurvedic medicinal wine (asava)

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Fermentative production of benzoic acid is seldom reported and the present report serendipitously expressed it, though in small quantity (~30 mg/l). Lipoxygenases (LOXs) catalyse the stereo-specific insertion of molecular oxygen into arachidonic acid, leading to hydroxy derivatives as end products. Serendipitously found LOX inhibitor was benzoic acid, produced in Ayurvedic-fermented medicated wine, pippalyasava. The present study reports the significance of the polyphenol derivatives in a traditional Ayurvedic therapeutic preparation where LOX inhibition might have a role, besides its importance in fermented benzoic acid production.

Keywords: Ayurvedic-fermented wine, benzoic acid, inflammation, LOX inhibitors, polyphenol metabolites.

THE lipoxygenases (LOXs), a family of non-haeme iron-containing dioxygenases that catalyse the stereospecific insertion of molecular oxygen into arachidonic acid¹, are classified with respect to their positional specificity of arachidonic acid oxygenation. They are referred to as 5-, 8-, 12- and 15-LOX^{2,3}. The biological properties of human lipoxygenases (hLOXs) have been extensively studied due to their involvement in several diseases such as bronchial asthma, allergic rhinitis, inflammatory skin diseases, rheumatoid arthritis (5-hLOX)⁴, cancer, osteoporosis, cardiovascular diseases (15-hLOX)^{5,6}, breast cancer and psoriasis (12-hLOX)^{7,8}.

Treating of different inflammatory conditions⁹⁻¹¹ involves the use of agents such as aspirin-like nonsteroidal anti-inflammatory agents, glucocorticoids, methotrexate and cyclophosphamide. These agents generally produce side effects, and are not suitable for chronic inflammatory symptoms^{12,13}. Hence, new agents, free of side-effects for treating inflammatory conditions are needed.

The present search for new LOX inhibitors has led to the serendipitous identification of benzoic acid produced in Ayurvedic-medicated wine, pippalyasava. The present study reports the significance of the polyphenol derivatives reports in a traditional Ayurvedic therapeutic preparation,

where LOX inhibition might have a role. Since the fermentative production of benzoic acid is industrially significant¹⁴, we report here the details of its serendipitously observed presence in the Ayurvedic fermentation¹⁵.

The LOX inhibition assay was performed with the methanolic extract of pippalyasava¹⁵. The inhibitory activity was measured by a modified spectrophotometric method¹⁶. LOX (EC 1.13.11.12) type I-B (Soybean), linoleic acid and all other compounds were purchased from Sigma (Sigma-Aldrich, UK) and used without further purification.

Enzyme solution of 0.93 μ M and substrate, linoleic acid solution of 0.32 mM were prepared in 0.2 mM borate buffer of pH 9.0. Purified compound was prepared at a concentration of 20 μ M. The assay mixture was made of 50 μ l of LOX and 360 μ l of the substrate. The final volume was made up to 2 ml with 1.59 ml borate buffer. The activity of LOX was measured by the formation of hydroperoxy eicosatetraenoic acid which was monitored at 234 nm on a spectrophotometer, HITACHI U 2900.

Thin layer chromatography was performed using silica gel 60 F254 plates (200 μ m, Merck, Germany) and CAMAG-UV visualizer. Column chromatography was conducted on silica gel 60 (60–120 mesh). HPLC was carried out on Shimadzu instrument using Phenomenex C18 250 \times 4.6 mm column. The mobile phase used was acetonitrile:1% glacialacetic acid in water (9:1) and detection at UV 280 and 343 nm. NMR spectral analyses and automated single crystal diffractometer (Bruker Kappa Apex II. ShellX structure solution package) were carried out for the structure elucidation.

¹³C-NMR were recorded on BRUKER NMR spectrometer with CDCl₃ as internal standard. ¹³C NMR spectra were measured at 400 MHz. IR spectra in KBr disk were recorded on Shimadzu 8900 FTIR spectrophotometer. LC separation was performed on a Waters AQUITY UPLC system (Waters Corp., Milford, USA) using AQUITY BEH C18 (100 mm \times 2.1 mm \times 1.7 μ m) column. Injection volume 10 μ l and isocratic elution by acetonitrile and 0.1% formic acid in water in the ratio 90:10 at a flow rate of 0.4 ml/min were given.

The Ayurvedic fermented herbal wine, pippalyasava was analysed for the presence of LOX inhibitor. A fraction responsible for the LOX inhibitory activity (Figure 1) was separated. The compound separated from the LOX inhibitory fraction and analysed by single crystal X-ray diffraction method, was benzoic acid ([see Supplementary data I online](#)). The NMR spectra ([see Supplementary data II, III and IV online](#)) peaks at 7.5 and 7.6 indicated the aromatic moiety, and in between 8 and 9 showed the phenolic OH proton. The FTIR spectra ([see Supplementary data V online](#)) peak in 3000 and above range represented the C–H linkage. Peak at 1701 indicated the carboxylic acid. The MS data also supported these findings ([see Supplementary data VI; Figure S1 online](#)). Peak at 77 showed

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the breakage peak of benzene ring. Peaks at 105 and 122 showed the corresponding breakage of CO and OH groups. NMR and IR analyses also showed that the compound responsible for the LOX inhibition was benzoic acid, a known LOX inhibitor¹⁷.

For finding out whether the compound benzoic acid is a degradation product of fermentation or not, we compared the HPTLC fingerprint (see [Supplementary Data VII online](#)) of methanolic extract of starting materials of pippalyasava with the methanolic extract of the fermented pippalyasava. The HPTLC fingerprint confirmed that the compound benzoic acid was a fermentation product.

The starting materials of pippalyasava, prior to fermentation, did not show the presence of benzoic acid, as evidenced from LC MS analysis (see [Supplementary data VI. Figure S2 online](#)).

In short, the results showed the fermentative production of benzoic acid by Ayurvedic fermentation protocol, which is significant industrially as well as medicinally. Fermentative production of benzoic acid is seldom reported¹⁴. The Ayurvedic fermentative protocols consist of highly complex input materials and microbial consortia¹⁵. Apart from the evidences presented above for the fermentative production of benzoic acid, at present, we are unable to present its line of production because of the complex nature involved in the process *per se*. However, the present report shows the fermentative production of benzoic acid. Fermentation of pippalyasava produced an average of ~30 mg/l benzoic acid. This is six times higher than an earlier reported benzoic acid production in wine without adding any specific substrate¹⁸.

This study is related to the fermentative production of benzoic acid which is industrially very significant. Also, since it may be present in fermented herbal medicines, such fermented products may deliver higher therapeutic effects mediated through LOX inhibition. In the recent

past, the anti-inflammatory properties of natural products or ingredients of traditional medicines and health foods and their specific compounds have been extensively studied for their therapeutic significances. However, the solubility of such compounds, in most cases is limited. Owing to their poor solubility, it is questionable whether their bioavailability could be accounted for their pharmacological effects. However, the polyphenols in health foods or drinks are readily metabolized to phenolic acids and aldehydes by the microflora of the intestines. This increases the possibility that such metabolites, rather than the original natural products or food ingredients, might be responsible for their anti-inflammatory properties¹⁹. However, in the case of fermented herbal medicines, the fermenting organisms may mimic the role of gut flora to the extent of modifying the compounds present in polyherbal drugs to deliver better soluble metabolites and enhance their therapeutic potentials^{20,21}. The anti-inflammatory property of Ayurvedic therapeutic products, particularly medicated wines, might be partly due to the derivatives of polyphenols present in them. The presently expressed production of benzoic acid by fermentation may be significant only in therapy. However, the principle involved, together with the microorganisms, could be exploited in the industry also for fermentative production of benzoic acid.

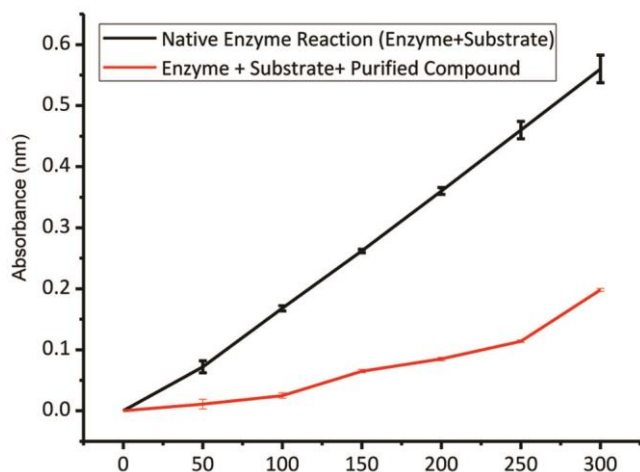


Figure 1. Graph showing the lipoxigenase inhibitory effect of purified compound (benzoic acid, average of six sets of experiments) from methanolic extract of 'Pippalyasava'.

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***In silico* characterization of ferritin-1 chloroplast targeting peptide encoding sequence in chickpea and pigeonpea**

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Chloroplast is an important organelle and a hub of photosynthetic reactions that occur in all plants. Majority of the chloroplast proteins are encoded by the nucleus and then transported into the chloroplast. The chloroplast transport is aided by leader sequence, chloroplast targeting peptide (cTP) that directs the proteins into the chloroplast. We identified the genes encoding chloroplast targeting proteins from chickpea (*Cicer arietinum*) and pigeonpea (*Cajanus cajan* and *Cajanus cajanifolius*) genomes. All the sequences were aligned, conserved motifs were identified and their phylogeny was deciphered. In this study, structural and functional features of chloroplast transit peptides of Ferritin-1 and its putative role in chloroplast targeting have been examined. The study would impart better understanding of targeting heterologous proteins to chloroplasts in pulses.

Keywords: Chloroplast targeting peptide, domains, ferritin-1, motifs, transgenes.

THE chloroplast is the site of photosynthesis that supports the life of most of the living organisms. Chloroplast targeting has emerged as an important tool for heterologous expression of transgenes in plants. The targeting of transgenes into the chloroplast by using a suitable strategy with an efficient expression cassette containing all the required elements has been the need of the day. Majority (90%) of the 3000 different proteins necessary for fully functional chloroplasts are known to be encoded by nuclear DNA. These proteins are synthesized in their precursor forms with an amino-terminal signal peptide called the transit peptide¹. A signature peptide sequence (chloroplast target peptide) drives protein into chloroplast and gets cleaved off after reaching target destination, delivering the protein to its destined cellular compartment². Chloroplast targeted expression of genes such as anthranilate synthase for increasing tryptophan production in maize has been achieved using novel constructs encoding chloroplast transit peptide³. Looking into the importance of cTP sequences in localizing transgenes, this study is based on the isolation of three cTP candidate sequences from chickpea and pigeonpea genome and their structural characterization.

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