

Evaluation of in vitro lipoxygenase Inhibition and Antioxidant Activity of Polyherbal Formulation Entoban

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ABSTRACT

Lipoxygenases are family of iron containing dioxygenases that convert the addition of molecular oxygen to fatty acid containing a cis-1, 4- pentadiene system. The primary product of this reaction is a 4-hydroperoxycis trans-1, 3-conjugated pentadienyl moiety within unsaturated fatty acid. This assay measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase with linoleic acid as substrate. When formulations of Entoban syrup and capsules were compared at various concentrations (10, 50 and 100 µg/ml), lipoxygenase inhibition activity increased in a dose dependent manner for both formulations just like standard bacilein. The formulations under test have good potential of lipoxygenase inhibition and prospective to be used in the treatment of various complications produced by lipoxygenase enzymes.

Keywords: *Lipoxygenase inhibition, inflammatory diseases, polyherbal, antioxidant.*

INTRODUCTION*

Lipoxygenases (LOXs) consist of a family of non-heme iron-containing dioxygenases, symbolizing the key enzymes involved in biosynthesis of leukotrienes (LTs) and catalyses the primary steps in the conversion of arachidonic acid to biologically active LTs (1). LTs are deemed as compelling mediators of hypersensitivity and inflammatory reactions (2). Several studies have shown that LTs

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may play a significant role in development of pathological conditions including kidney stones, pyelonephritis, peptic ulcer and other inflammatory diseases of digestive tract (3). Concerning their pro-inflammatory possessions the inhibition of 5-lipoxygenase pathway is believed to be remarkable in the management of inflammatory diseases (4). The distinctive function of the enzyme 5-lipoxygenase in the production of LTs makes it a probable goal for biochemical manipulation. Owing to the increase production of LTs concerned in several inflammatory diseases, there has been

substantial interest in the generation of 5-LO inhibitors intended for therapeutic purpose. The compounds recognized as 5-LO inhibitors can be divided into antioxidants, substrate-analogous, and miscellaneous grouping of inhibitors. The underlying principle for employing 5-LO inhibitors for the management of gastrointestinal diseases is based on the increased production of LTs in the inflamed mucosa, LTB₄ being the most compelling chemotactic and chemokinetic metabolite of arachidonic acid (5).

Though exercise of using numerous anti-inflammatory drugs is in vogue, the persistent use of these for an extended period of time can have unfavorable side effects. Hence, there is need to explore substitution approaches to decrease the production of inflammatory mediators with natural dietary products. Phytomedicine has demonstrated to be an intact treasure for the innovation of model compounds to treat diseases of various etiologies (6-8). Many phenolic/flavonoid compounds originated from vegetables source are revealed to modulate 5-LO and prostaglandin H synthase pathways of arachidonic acid. Several natural and synthetic compounds with redox and non-redox potential are identified as inhibitors of 5-LO (5). Herein we report the Lipoxygenase inhibition activity of polyherbal formulation Entoban.

EXPERIMENTAL

Composition of capsule

Each 500mg capsule contains:

Holarrhena antidysenterica (400 mg), Myrtus communis (400 mg), Symplocos racemosa (200 mg), Aluminum silicate (200 mg), Quercus infectoria (100 mg), Zingiber officinalis (100 mg), Helicteres isora (100 mg), Berberis aristata (100 mg), Butea frondosa (100 mg), Aegle marmelos (100 mg), Acacia Arabica (100 mg).

Composition of Entoban syrup

Each 10 ml contains:

Aegle marmelos (Syrup; Oral; 100 mg), Berberis aristata (Extract 30 mg), Butea frondosa (Dry extract 20mg), Holarrhena antidysenterica (Dry Extract 50mg), Myrtus communis (Dry Extract 200 mg), Quercus infectoria (Dry Extract 50 mg).

Plant Material

Herbs used in Entoban syrup and capsules were stored in dark at 23°C. All herbs were tested for their prescribed part, macro and microscopic descriptions.

Preparation of plant extract

Individual grinded herbs were taken into extractor and water using as solvent was added to the grinded herbs in the ratio of 1:10. The extractors were heated with steam for 2 - 3 hours to obtain the required extract in the form of decoction (individual liquid extract). The decoctions were then filtered and shifted to evaporators to get rid of the extra solvent and to get the required moisture content i.e. not more than 25%. The individual extracts were stored in the form of thick extracts.

Chemicals and reagents

Linoleic acid and lipoxygenase were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical reagent grade from Merck. Cadmium chloride (CdCl₂), Dalbeco Eagle's Minimum Essential Medium (D-MEM), Dimethyl sulphoxide (DMSO) and Ethylene diamine tetra acetic acid (EDTA) from Wako Pure Chemical Industries Ltd. and Trypsin (0.25%) from Gibco, Canada.

Lipoxygenase Inhibition Activity

1. Lipoxygenase enzyme solution was prepared in sodium phosphate buffer with such concentration to give 130 U per well.
2. Sodium phosphate buffer (pH 8.0: 160µl:100

mM) was taken in each well of plate labelled as Blank (Bsubstrate and Benzyme), Control and Test.

3. Test compound solution in methanol (10-1000 μ M: 10 μ l) was added in each well labeled as test.
4. Lipoxygenase solution (LOX: 20 μ l) was added in each well including B enzyme, Control and Test except Bsubstrate and the mixture was incubated at 25 μ C for ten minutes.
5. Substrate solution was prepared by adding linoleic acid (155 μ l:0.5 mM) into 0.12 % w/v tween 20 (257 μ l). The mixture was mixed and 0.6 ml NaOH (1N) was added to remove turbidity and volume was making up to 20 ml with deionized water. This mixture was flushed with nitrogen gas to avoid autoxidation before adding to each well.
6. The reaction was initiated by the addition of 10 μ l substrate in each well except B (enzyme) and the absorbance was measured after five minutes at 234 nm.

Cell line and culture

HepG2 cells originally derived from human hepatocellular carcinoma were procured from Riken, Japan. The cells were grown in 10 cm culture plate containing D-MEM supplemented with 10% FBS, L-glutamine and phenol red at 37°C in a CO₂ incubator (SANYO, Japan) in an atmosphere of humidified 5% CO₂ in 95% air. The cells were then trypsinized to seed in the 96 well-plate for the experiment.

Cell Viability Assay

Cell viabilities in response to polyherbal formulations on the HepG2 cells were measured to check any cytoprotective response and antioxidant activity of the drugs by using cell counting kit 8 (CCK-8) (Dojindo, Japan). The

CCK-8 is made up of Dojindo's highly water-soluble tetrazolium salt, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt]; which produces a water-soluble, yellowish formazan dye upon reduction in an electron carrier (9). The number of viable cells is directly proportional to the amount of the formazan dye generated in cells. Assays were done following manufacturer's instruction. Briefly, cells were seeded at density of 5000 cells per well in 96-well plates in a triplicate and pre-incubated overnight for adherence at physiological conditions of 5% CO₂ and 37°C, in a humidified atmosphere. The cells were then pre-treated at concentrations of 0 μ g/ml (NT), 100 μ g/ml, 200 μ g/ml, 300 μ g/ml, 400 μ g/ml, 500 μ g/ml from both formulations; capsule and syrup of Entoban separately in two groups; one without cadmium and one with cadmium. Here cadmium was used to induce oxidation in the HepG2 cells and one sample with only cadmium as a positive control. After 12 hours incubation, add 25 μ M cadmium and incubated again for 24 hours. After incubation, 10 μ l of CCK-8 was added to each well including blank sample and incubated at 37°C for 3 hours; followed by the measurement of absorbance using an automated micro-plate reader ELx 800 (BioTek, UK) at 450 nm.

RESULTS

When formulations of Entoban syrup and capsules were compared at various concentrations (10, 50 and 100 μ g/ml), lipoxygenase inhibition activity increased in a dose dependent manner for both formulations just like standard. (Table 1) However the Entoban syrup revealed better lipoxygenase inhibition activity as compared to Entoban capsules. At 10 μ g/ml syrup possess 31.2% whereas capsules possess 12.3% inhibition activity. At 50 μ g/ml syrup exhibit 45.6% inhibition activity whereas capsules 32.4%.

Table 1. Lipoxygenase Inhibition Activity of Entoban Syrup, capsules and standard

Concentration tested	Percent Activity (%) syrup	Percent Activity (%) capsules	Percent Activity (%) standard
10 µg/ml	31.2	12.3	61.3
50 µg/ml	45.6	32.4	73.4
100 µg/ml	67.3	45.6	85.3

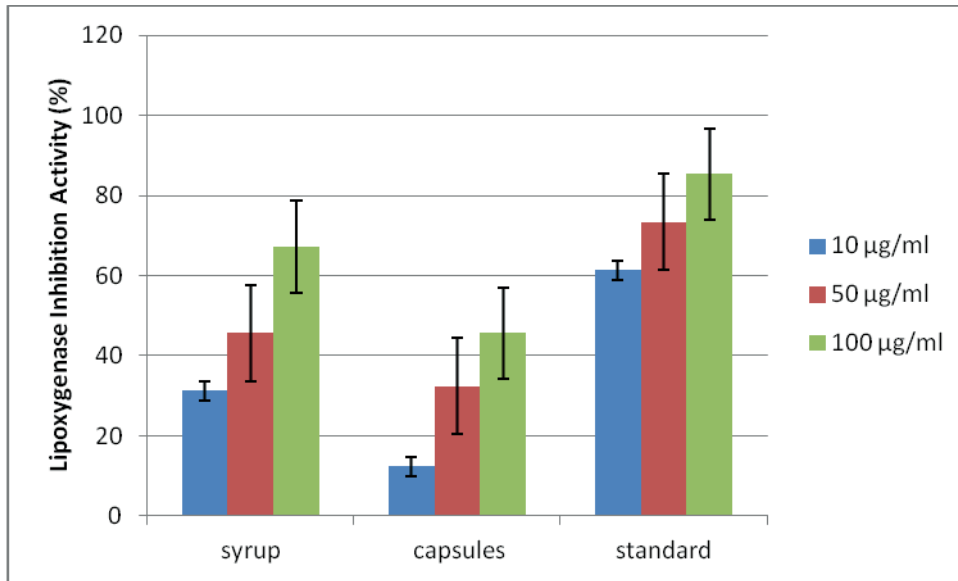


Fig. 1. Comparison of Lipoxygenase inhibition potential of Formulations with Standard Error

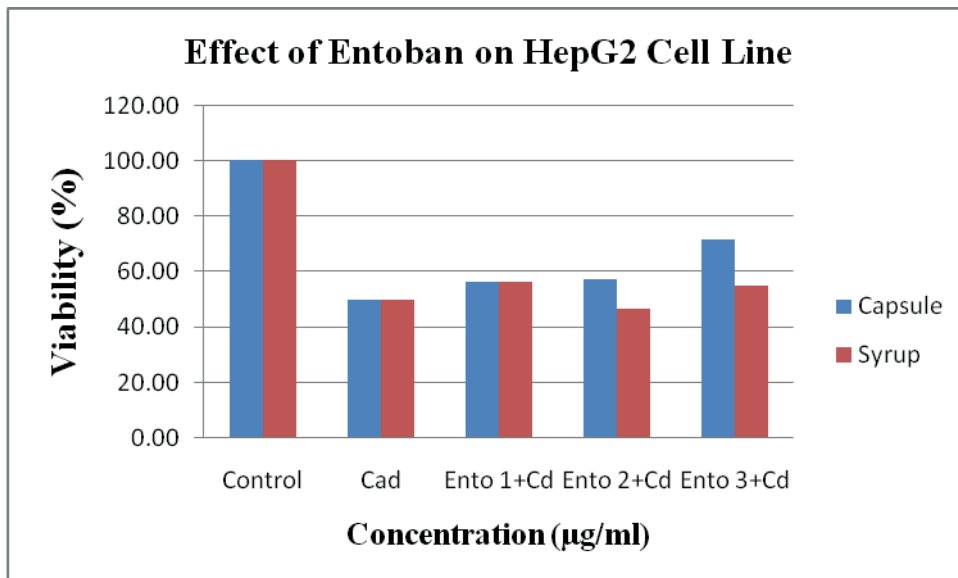


Fig. 2. Anti-oxidant effect of Entoban capsule and syrup on the HepG2 cell line by cck-8 cell viability assay. NT showed non-treated cells and Cd was used as a positive control and the drugs were examined in 3 different concentrations.

At 100 µg/ml syrup possess 67.3% inhibition activity whereas capsules possess 45.6% inhibition activity. The standard bacilein revealed 61.3%, 73.4% and 85.3% lipoxygenase inhibition activity at concentrations of 10, 50 and 100 µg/ml respectively. All these results showed that lipoxygenase inhibition activity increased in a dose dependent manner for both formulations. Results showed that both formulations of Entoban syrup and capsules have good lipoxygenase inhibition potential when compared to bacilein. (Figure 1).

Cell viabilities were measured by CCK-8 assay in the HepG2 cell line treated with different concentrations of the Entoban capsule and syrup separately after inducing the oxidation by a strong oxidative agent, cadmium. It was found that Entoban capsule can rescue the cell protection at the dose of 300µg/ml around 78-80% in the HepG2 cells (Figure 2).

DISCUSSIONS

Although there are splendid advancements in modern medicine, yet traditional medicine has always been accomplished for treating diseases of various etiologies. The traditional medicine sector has become an imperative resource in health care, particularly in rural and tribal areas of the country (10). The present study was directed to a polyherbal formulation Entoban which incorporates an outstanding combination of herbs i.e. *Holarrhena antidysenterica*, *Berberis aristata*, *Symplocos racemosa*, *Myrtus communis*, *Quercus infectoria* and *Helicteres isora*. Berberine, the biomarker of *Berberis aristata* was reported to exhibit a range of pharmacological and biological activities, and interest has been focused on its antioxidative potential (11-12). The activity of 1-ethyl brachiose-3'-acetate and triacetyl palmitate present in *Symplocos racemosa* displayed the inhibitory potential against lipoxygenase enzyme in a dose dependent manner (13).

Research has shown that owing to the active constituents of galls of *Quercus infectoria* comprises a large amount of tannins, the activity of the *Quercus infectoria* the aphthous powder and aphthous gel could inhibit the production of the inflammatory mediators such as IL-6 and PGE2 (14). The present research confirms the similar findings that both formulations of Entoban executing invitro lipoxygenase inhibition due to the presence of herbs depicting such activities. Enzyme inhibition is a significant area of pharmaceutical research leading to the innovations of drugs having remarkable performance in diverse physiological conditions. Lipoxygenases are family of iron containing dioxygenases that convert the addition of molecular oxygen to fatty acid containing a cis-1, 4- pentadiene system. The primary product of this reaction is a 4-hydroperoxycis trans-1, 3-conjugated pentadienyl moiety within unsaturated fatty acid. This assay measures the hydroperoxides produced in the lipoxygenation reaction using a purified lipoxygenase with linoleic acid as substrate (15). When formulations of syrup and capsules were compared at various concentrations, lipoxygenase inhibition activity increased in a dose dependent manner for both formulations just like standard revealing that both formulations of syrup and capsules have good anti lipoxygenase potential. However the Entoban syrup revealed better lipoxygenase inhibition activity as compared to Entoban capsules.

It was also found that Entoban capsule at the dose 300µg/ml concentration has cytoprotective activity (approx. 76%), to rescue the cell viability after induction of apoptosis by using strong oxidant agent, cadmium. Previous studies conducted in vivo and in vitro also indicated that similar plants used in the formulation of capsule and syrup, shown anti-oxidant and hepatoprotective effects (16-17).

CONCLUSIONS

The formulations under test have an excellent potential of lipoxygenase inhibition, antioxidant and cytoprotective effects. It is expected that this formulation could be possibly used in the treatment of various complications produced by lipoxygenase enzymes.

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Conflict of Interest

All authors have declare no conflict of interest

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