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Research Article

Effect of Hydro Alcoholic Extracts of *Boswellia serrata* and *Terminalia bellerica* Against Cyclo-Oxygenase and Lipoxygenase Enzymes- An *In Vitro* Approach

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ABSTRACT

Introduction: Inflammation is a key mediator in plenty of important physiological abnormalities like cancer, myocardial infarction and arthritis. Natural anti-inflammatory agents are of great demand owing to its decreased side effects and increased curative properties. Aim: To investigate the *in vitro* inhibitory effect of three different concentrations of hydroalcoholic extract of *Boswellia serrata* (BS) and *Terminalia bellerica* (TB) on COX-2 and LOX-2 enzymes. Materials and Methods: Three different concentrations of hydroalcoholic menstruum of BS and TB were prepared using the standard procedures. The *in vitro* inhibitory activities of COX-2 and LOX-2 were performed using previously mentioned methods with minor modifications. Results: Among the different concentrations used the 90:10 menstruum of BS at 12 mg/ml displayed maximum inhibitory potential against COX-2 enzyme when compared to the same concentration of TB extract. This is suggestive that the majority of bioactive compounds present in BS was alcohol soluble and hence elicited maximum inhibition potential at 90 % ethanol ratio. A similar pattern of results were observed against LOX-2 enzyme as well. Conclusion: From the results it is concluded that the 90:10 hydroalcoholic extract of *Boswellia serrata* and *Terminalia bellerica* exhibit appreciable percentage of COX-2 and LOX-2 inhibition, which could be attributed to the ethanol soluble bioactive components present in the extract.

Keywords: Inflammation, *Boswellia serrata*, *Terminalia bellerica*, cycloxygenase activity, Lipoxygenase activity

INTRODUCTION

Boswellia serrata is one of the most valuable medicinal plants used in ayurvedic medicine and also the extracted active components are key ingredients in allopathic medicines. The exudates from this family (Burseraceae) of tree called *Salai*, an oleo gum-resin, is collected by making incision made on the trunk of the tree, which is then stored in a specially made containers. Earlier research indicates that the major components of the exudates constitute resin and essential oils in the ranges of 30-60% and 5-10%, respectively[1]. Interestingly this resin and oils are soluble in organic solvents. The remaining part of this exudates is made up of polysaccharides (65% arabinose, galactose, and xylose) which are water soluble. The key phytochemicals of the resinous part is identified as monoterpenes (α-thujene), diterpenes (incensole, incensole oxide, iso-incensole oxide), a diterpene alcohol (serratol), triterpenes (α- and β-amyrins), pentacyclic triterpenic acids (boswellic acids), tetracyclic triterpenic acids (tirucall-8,24-dien-21-oic acids) and many more to be listed[2&3]. It has been proved that the clinical trials of gum-resin of *Boswellia serrata* has displayed improved symptoms in osteoarthritis and rheumatoid arthritis patients[4]. In addition to this, other researchers have summarized the ameliorative actions of boswellic acids extracted from the same tree on the molecular level of animal trials and human patients suffering from inflammation and cancer[5]. Despite these two roles there are plenty of other available research data suggesting importance of this tree.

Terminalia bellerica belongs to the family of Combretaceae is also a well-known tree used for folklore potential uses. Eventhough various parts of the tree contains different action levels, the fruits of this tree are massively used because of its multifaceted herbal implications such as laxative, astringent, anti-helmintic and antipyretic, treatment of hepatitis, bronchitis, asthma, dyspepsia, piles, diarrhea, coughs, hoarseness of voice, eye diseases and scorpion-sting. Kernel of the fruit is useful as a narcotic as well as has purgative action and chronic usage is well tolerated in mice[6]. The crude extracts from various parts of this plant have been fractionated and purified and the results identified that the extract possesses constituents such as glucoside, gallo-tannic acid, essential oils, ellagic acid, gallic acid, lignans, tannins, ellagic acid, ethyl gallate, galloyl glucose and chebulic acid, phyllemblin, β-sitosterol mannitol, glucose, fructose and rhamnose[7]. These compounds might be the responsible factor for the pharmacological activities such as antimicrobial,

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Table 1: Effect of different solvent ratios on the yield percentage of *Boswellia serrata* and *Terminalia bellerica*.

S.No.	Extract	Yield percentage
1	90:10 Hydroalcoholic extract of BS resin	10.5
2	70:30 Hydroalcoholic extract of BS resin	13.3
3	50:50 Hydroalcoholic extract of BS resin	15.2
4	90:10 Hydroalcoholic extract of TB *dried fruit*	15.1
5	70:30 Hydroalcoholic extract of TB *dried fruit*	16.0
6	50:50 Hydroalcoholic extract of TB *dried fruit*	17.3

antioxidant, hepatoprotective, antispasmodic and bronchodilatory activities.

As described above the two plants contains significant amount of phytochemicals. But in general the ability of these phytochemicals to confluence in a single solvent with a defined polarity is difficult as they differ widely based on the charges/polarity. However, from previous researches it is evident that the ethanol and methanol serves as better organic solvents to isolate fairly all phytochemicals. Interestingly, when these solvents in an appropriate composition mixed with water become hydroalcoholic extract, which is more potent solvent for the phytochemical oriented studies.

Cyclo-oxygenase (COX) are lipid metabolizing enzymes that catalyzes the oxygenation of polyunsaturated fatty acids (PUFA) using arachidonic acid as a starting material to generate the prostanoids that are essential for cell-signaling molecules associated with the initiation, maintenance and resolution of physiological inflammatory processes. The two isoforms of these enzymes have been identified such as COX-1 and COX-2, and it has been postulated that while COX-1 is expressed in mammalian cells, particularly in endothelium, platelets, and kidneys in physiological conditions, COX-2 is mainly inducible in pathological conditions by inflammatory stimulation[8]. Selective modulation of many prostanoids has important therapeutic potential for the treatment of inflammation and inflammatory conditions such as rheumatoid arthritis[9]. Lipoxygenase (LOX) interaction products or Lipoxins are yet another group of lipid mediators formed during arachidonic acid metabolic pathway[10]. They are generated during the cellular interactions that occur as part of the multicellular host response to inflammation. They are produced not only by the 5-LOX pathway, but also by the action of two other enzymes such as 12-LOX and 15-LOX. Similar to the COX enzyme, any compound capable of interfering with the LOX enzymes are also a critical clinical significance against inflammatory process.

In line with all these observations, an attempt has been made to investigate the inhibitory effect of three different concentrations of hydroalcoholic extract of *Boswellia serrata* (BS) and *Terminalia bellerica* (TB) on COX-2 and LOX-2 enzymes.

MATERIALS AND METHODS

Chemicals

All chemicals used in the present study were of analytical grade.

Preparation of the extracts

The resin of BS and dried fruit material of TB were collected from the raw drugs department of Arya Vaidya Sala, Kottakkal.The TB was grounded and made into coarse powder. Later, 100 g of the resin from BS and same amount of TB fruit powder was extracted in 600 ml of hydro-alcoholic mixture using Soxhlet apparatus for 8 h. The residue obtained is collected in a pre-weighed beaker and the remaining any excess solvent is evaporated in a rotary flash evaporator and total yield is calculated. The ratio of aqueous and ethanol used for extraction is mentioned below. Each of the different solvent ratios were used separately in 100 g of samples and not extracted sequentially. The 90:10 hydro-alcoholic mixtures were made by adding 90 ml of ethanol + 10 ml of distilled water.

90:10 Hydroalcoholic extract of BS resin
70:30 Hydroalcoholic extract of BS resin
50:50 Hydroalcoholic extract of BS resin
90:10 Hydroalcoholic extract of TB *dried fruit*
70:30 Hydroalcoholic extract of TB *dried fruit*
50:50 Hydroalcoholic extract of TB *dried fruit*

Determination of cyclo-oxygenase inhibitory activity

This assay was done using the previously mentioned methods with minor modifications[20]. Each of the collected extracts was made to a stock concentration of 20 mg/ml using distilled water. The samples and positive control (naproxen) were taken in the concentrations of 0.1, 0.3, 0.6, 0.8,1, 1.5, 2, 3, 6, 12 mg/ml, respectively. The samples as well as the positive control were pre-incubated with the enzyme-containing reaction buffer for 1 min prior to the addition of arachidonic acid (AA). A separate test tube with all the reaction mixtures added except the test sample was treated as blank. The assay was performed using 100 IU of ovine COX-2 (one unit of enzyme is nmol of oxygen consumed per minute at 37°C) in 0.1MTris- HCl buffer (pH-8) containing 20 µM AA, 5 mM EDTA, 2 mM phenol, and 1 µM hematin. The reaction was initiated by the addition of 20 µM AA. Final OD was measured at 502 nm using an UV spectrophotometer. The percentage of COX-2 inhibition was statistically analyzed using Origin pro-8 software (Version 3.6).

Determination of lipoxygenase inhibitory activity

Lipoxygenase inhibiting activity was conveniently measured by slight modification of the previously mentioned method[20]. Each of the collected extracts was made to a stock concentration of 20 mg/ml using distilled water. The samples and positive control (naproxen) were taken in the concentrations of 0.1, 0.3, 0.6, 0.8, 1, 1.5, 2, 3, 6, 12 mg/ml, respectively. Added160 ml of 0.1 mM sodium phosphate buffer (pH-7), 10 ml of the sample solution and 20 ml of Lipoxygenase and mixed well and incubated for 5 min at 25°C. The reaction was initiated by the addition of 10 µl linoleic acid solution and the

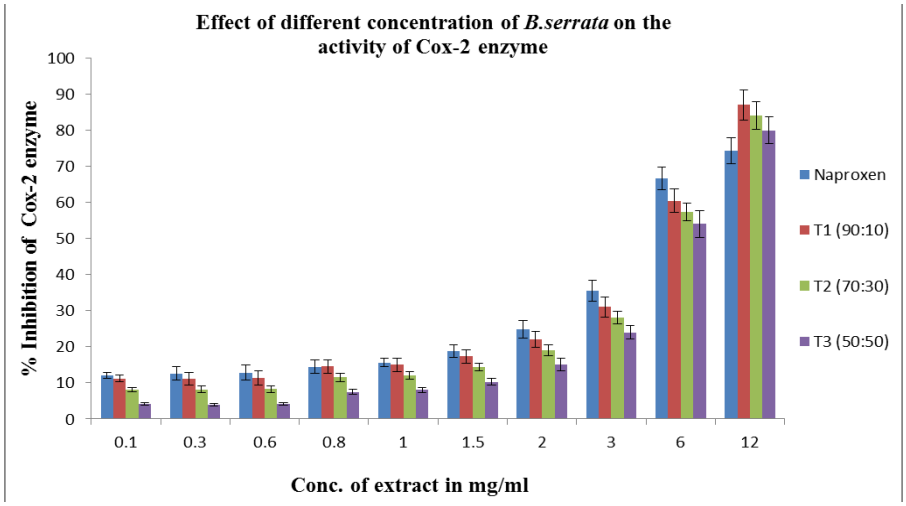

Figure 1: Effect of different concentrations of BS extract on COX-2 enzyme activity.

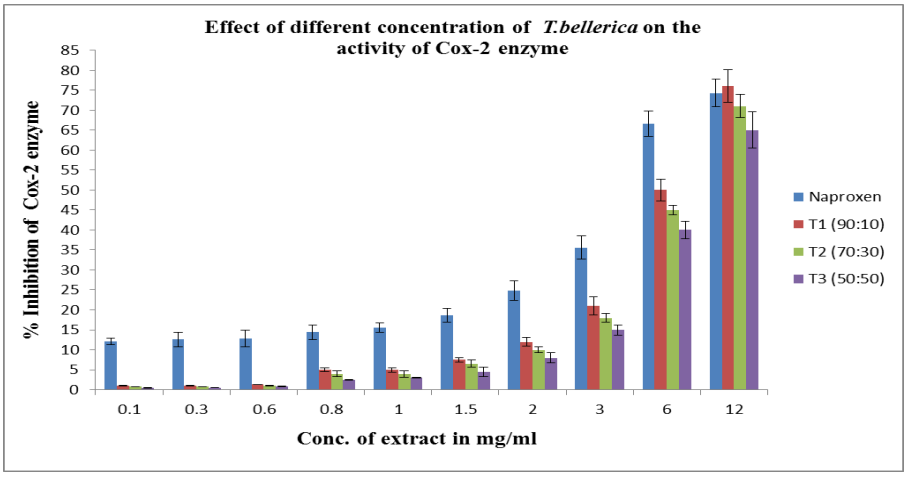

Figure 2: Effect of different concentrations of TB extract on COX-2 enzyme activity.

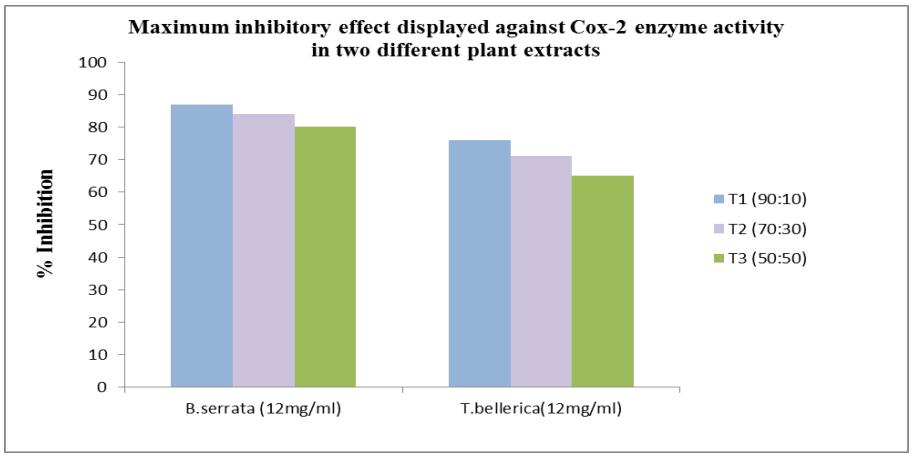

Figure 3: Comparative graph showing the maximum inhibitory concentrations of TB and BS extract on COX-2 enzyme activity.

absorption change with the formation of (9Z, 11E)-13S-13-hydroperoxyoctadeca-9, and 11-dienoate. The changes in the absorbance were measured at 234 nm using a spectrophotometer. The percentage of LOX-2 inhibition observed was statistically analyzed using Origin pro-8 software(Version 3.6).

Statistical analysis

The data are expressed as mean ± SD from three independent experiments. The statistical analysis were calculated using Microsoft excel software (2007) for Windows operating system.

RESULTS

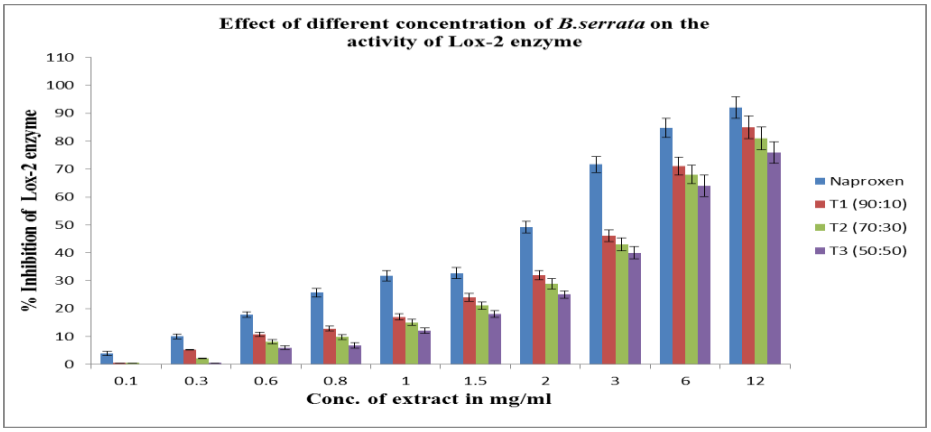

Figure 4: Effect of different concentrations of BS extract on LOX-2 enzyme activity.

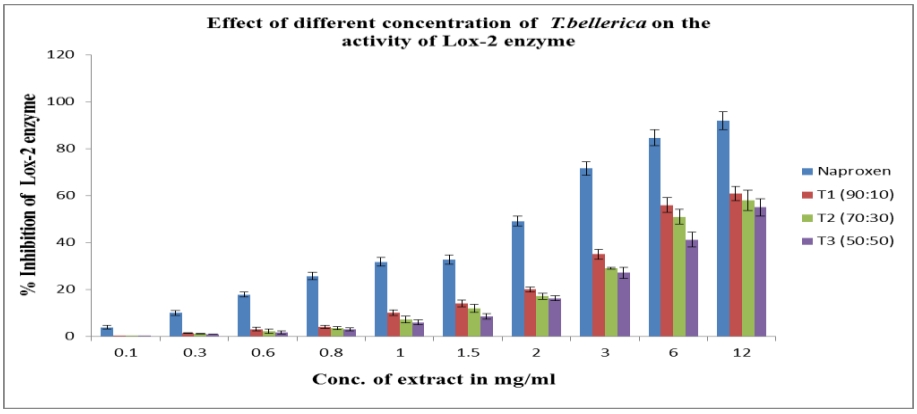

Figure 5: Effect of different concentrations of TB extract on LOX-2 enzyme activity.

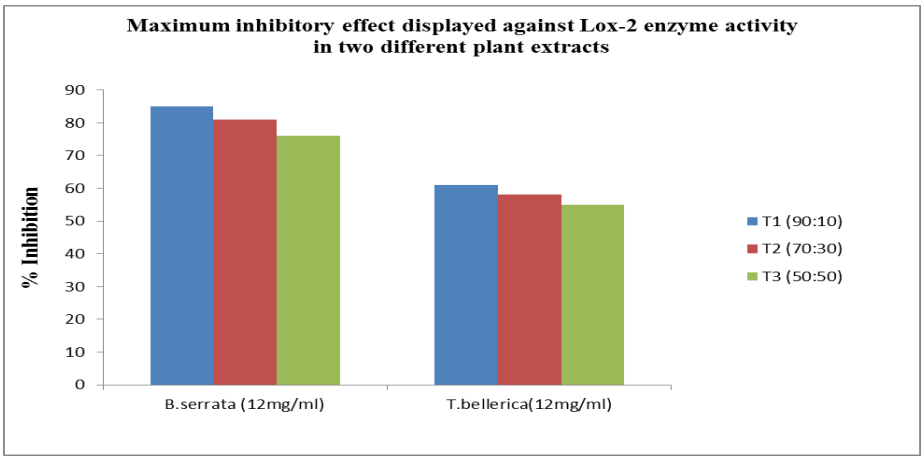

Figure 6: Comparative graph showing the maximum inhibitory concentrations of TB and BS extract on LOX-2 enzyme activity.

Effect of different solvent ratio on the yield percentage of BS and TB

The effect of changes in the composition of water to alcohol content has significant impact in the contribution of the yield percentage. Accordingly, in the present study the maximum yield was obtained from TB at 50:50 ratios of hydroalcoholic contents, whereas, the minimum percentage was obtained from BS whilst the usage of 90:10 ratios of hydroalcoholic solvent. The yield percentages of other solvent ratios are displayed in Table: 1.

Effect of different concentrations of BS extracts on the activity of COX-2 enzyme

The results of the study using different concentrations of hydro-alcoholic extracts of BS on COX-2 enzyme are displayed in Fig.1. The three different hydroalcoholic concentrations are indicated as T1, T2 and T3 inside the figure. The results indicate that a concentration-dependent increase in the enzyme inhibition was observed in all the three concentrations used. The T1 extract of BS displayed maximum inhibitory potential in all the concentrations used when compared to T2 and T3. Eventhough, the

inhibitory percentage of standard drug Naproxen elicited a dose-dependent increase to inhibit COX-2 enzyme, the observed activity is lesser when compared to the other extracts at the concentration of 12 mg/ml.

Effect of different concentrations of TB extracts on the activity of COX-2 enzyme

The results of the study using different concentrations of hydro-alcoholic extracts of TB on COX-2 enzyme are displayed in Fig.2. The three different hydroalcoholic concentrations are indicated as T1, T2 and T3 inside the figure. The results indicate that a concentration-dependent raise in the enzyme inhibition of COX-2 was observed in all the three concentrations used. However, first three concentrations used such as 0.1, 0.3 and 0.6 mg/ml exhibited minimal inhibition when compared to other concentrations used. Interestingly, even in TB extracts the T1 ratio of solvent system displayed maximum inhibitory potential in all the ranges of concentration used. On the other hand, the T1 extract from TB at 12 mg/dl showed raised COX-2 inhibition than the standard drug used at the same concentration. A comparative graph showing the relative inhibition percentages of 3 different extracts of BS and TB are displayed in Fig.3. The results clearly depicts that BS extract has significant *in vitro* inhibition potential than the TB extract at all the solvent ratios used (T1, T2 & T3).

Effect of different concentrations of BS extracts on the activity of LOX-2 enzyme

The results of the study using different concentrations of hydro-alcoholic extracts of BS on LOX-2 enzyme are displayed in Fig.4. The three different hydroalcoholic concentrations are indicated as T1, T2 and T3 inside the figure. The results indicate that a concentration-dependent increase in the enzyme inhibition was observed in all the three concentrations used. The T1 extract of BS displayed maximum inhibitory potential in all the concentrations used when compared to T2 and T3. In contrast to above observed results, in this assay the naproxen standard excelled in inhibiting the LOX-2 enzyme than the plant extracts used at all the concentrations used.

Effect of different concentrations of TB extracts on the activity of LOX-2 enzyme

The results of the study using different concentrations of hydro-alcoholic extracts of TB on LOX-2 enzyme are displayed in Fig.5. The results indicate that a concentration-dependent raise in the enzyme inhibition of LOX-2 was observed in all the three concentrations used with minimal or insignificant inhibition from 0.1-0.6 mg/ml. Interestingly in TB extracts the T1 ratio of solvent system displayed maximum inhibitory potential in all the ranges of concentration used. In the current inhibition assay against LOX-2 enzyme, the standard drug showed massive difference in the inhibition potential or in other words the effect of the plant extracts were comparatively lesser when compared to the naproxen and also BS extracts added at the same concentrations. A comparative graph showing the relative inhibition percentages of 3 different extracts of BS and TB are displayed in Fig.6.

The results clearly depicts that BS extract has significant *in vitro* inhibition potential against LOX-2 enzyme than the TB extract at all the solvent ratios used (T1, T2 & T3). Lastly among the T1, T2 and T3, the T1 solvent system indicated better inhibition than the others i.e. a ratio of 90:10 hydroalcoholic extract has appreciable inhibitory potential against the COX-2 and LOX-2 enzymes.

DISCUSSION

In general the processes of wound heal occurring in the body with the lapse of time is a greatest gift of nature mother to mankind. During this process, the body responds/reflects this process through different modes such as pain, swelling, raised temperature and erythema. In order to reduce the incidence of this process allopathic doctors prescribe anti-inflammatory drugs called NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). Although these drugs provide temporary relief, research data suggests that it could give undesirable side effects such as gastric ulceration, liver damage and even stimulates the likelihood of getting myocardial infarction and stroke[16]. In this case, natural anti-inflammatory compounds are of immense interest and have been used to mediate the anti-inflammatory process often with lesser side effects[11]. As described earlier the combination of aqueous and alcoholic solvent plays a pivotal role in extracting the phytochemicals during the extraction process. The amount of yield depends on solvents, time and temperature of extraction as well as the chemical nature of the sample. Under the same time and temperature conditions, the polarity of solvent and chemical property of sample is the prime factors to determine the yield of the extract[14]. Thus, the present study reveals that a combination of 50 % of water and 50% of ethanol is best suited for the isolation of compounds from both the plants. This is evidenced from the maximum yields of 15.2 % and 17.3 % from BS and TB, respectively, when compared to the other fractions. Thus it is plausible that majority of phytoconstituents present in both the plants are aqueous as well as ethanol soluble in nature. The results are in concordance with previous study using aerial parts of *Momordica charantia*, wherein the yield percentage increased in 50% hydroalcoholic extract than the other solvents used[15].

The inflammatory process is a composite network of biochemical pathways, which is activated by an injury or microbial invasion or degenerative diseases, if not controlled, would lead to many unfavorable consequences in the body. A precursor material for the inflammatory pathway is the arachidonic acid because arachidonic acid is immediately released from injured cellular membranes and then metabolized into prostaglandins and leukotrienes with the involvement of COX-1/2 and LOX enzymes, respectively[12].The discovery of two isoforms of COX such as COX-1 and COX-2, has contributed to an improved indulgent of the cell mediators and effects triggered during an inflammatory response. As a result of this finding, plant-based inhibitors are now being intensively searched to simultaneously and selectively inhibit COX as well as LOX enzymes to prevent the inflammation mediated adverse reactions[13]. The results of the present investigation depicts that both of the plant extracts had shown appreciable *in vitro* inhibition against COX-2 enzyme.

Eventhough the total yield percentages was higher in 50 % alcoholic extract, the observed inhibition percentage was superior in the extracts collected using 90 % ethanol in both the plants while compared to the other extracts. Furthermore, among the 90:10 extracts of BS and TB, the BS extract displayed greater inhibition potential against COX-2 enzyme. Interestingly, the 90:10 menstruum of BS at the concentration of 12mg/ml, elicited higher inhibition potential than the effect indicated by naproxen standard used at the same concentration. Some common side effects of the synthetic drugs like naproxen, ibuprofen, and flurbiprofen include gastric irritation, ulceration, bleeding, renal failure, interstitial nephritis, hepatic failure, headache, thrombocytopenia, hemolytic anemia and asthma. Hence, it could be reasonably believed that the 90:10 extract of BS can be used an anti-inflammatory agent without encountering any of the above mentioned side effects. The results are in similar fashion to the *in vivo* studies of previous researchers[17].

It is a known fact that the inflammatory response is initiated by the activation of T-cells, chemokines, adhesion molecules, inflammatory cytokines, growth factors, colony stimulating factors and pro-inflammatory enzymes like COX and LOX. Any potent carcinogen could activate the NF-κB pathways which directly up-regulates the expression of LOX 2 and inflammatory cytokines that has significant effect in the carcinogenesis[18]. Thus inhibiting the LOX-2 enzymes is very vital for the inhibition of carcinogenesis and inflammation. The results of the present investigation portrays that both the plant extracts had shown fairly good *in vitro* inhibition against LOX-2 enzyme. Similar to the results of COX-2, even in LOX-2 inhibition assay, the maximum inhibition was observed in the 90:10 menstruums of BS/TB and the least inhibition was observed with 50% alcoholic extract of the same plants. In contrary to the COX-2 results, here in LOX-2 the percentage inhibitions displayed by plant extracts were relatively lesser while compared to naproxen standard. Among the BS and TB extracts, the activity of BS at 12mg/dl concentration was nearly equal to the inhibition percentage of naproxen at the same concentration. On the other hand, the TB extracts didn't indicate a significant inhibition at the 12 mg/dl. In a result that was observed in curcumin previously, the researchers were able to down-regulate different molecular pathways involved in inflammation thereby decreasing the generation of different inflammatory mediators such as LOX-2, iNOS, and cytokines, net effectively preventing the carcinogenesis process in several types of cancer[19]. Thus to put up the present results in a consolidated way, the 90% ethanolic extract has increased probability to inhibit the COX-2 enzyme than LOX-2. Though the percentage inhibition of LOX-2 enzyme in comparison to naproxen standard is not very promising, both the extracts still had displayed more than 50% inhibition for the LOX-2 enzyme consequently, an improved ability to reduce the event of inflammatory reactions.

CONCLUSION

From the results it is concluded that the 90:10 hydroalcoholic extract of *Boswellia serrata* and *Terminalia bellerica* exhibit appreciable percentage of COX-2 and LOX-2 inhibition, which is attributable to the bioactive components present in the extract. Further studies are warranted to isolate and characterize the bioactive components responsible for eliciting this action.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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